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Mono- and combination immunotherapy in dogs with spontaneous tumors

Stimulation of the adaptive and innate immune system against cancer and suppression of immunosuppressive immune cells

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List of Abbreviations

AA	=	acanthomatous ameloblastoma
ADCC	=	antibody-dependent cell-mediated cytotoxicity
APC	=	antigen presenting cell
ATP	=	adenosine triphosphate
BCG	=	bacillus Calmette-Guerin
CAR	=	chimeric antigen receptor
CBC	=	complete blood count
CC	=	cancer cell
CC-DC hybrid	=	cell formed throug merger between a CC and a DC
CCR5	=	chemokine receptor 5
CEP	=	circulating endothelial progenitor
CEUS	=	contrast-enhanced ultrasound
cIL-12	=	canine IL-12
CM	=	culture medium
CMV	=	cytomegalovirus
CP	=	cyclophosphamide
CR	=	complete response
CRT	=	calreticulin
CSC	=	cancer stem cell
CT	=	computed tomography
CTL	=	cytotoxic T lymphocyte
CTLA-4	=	cytotoxic T lymphocyte associated antigen 4
CXCL	=	CXC-motif ligand
DAMP	=	damage-associated molecular pattern
DC	=	dendritic cell
DC-CM	=	CM for DCs
DFI	=	disease-free interval
DMEM	=	Dulbecco modified Eagle's medium
DPBS	=	Dulbecco's phosphate-buffered solution
ECM	=	extracellular matrix
EGT	=	electrogene transfer
FACS	=	fluorescence-assisted cell sorting
FGF	=	fibroblast growth factor
FMO	=	fluorescence minus one
GMP	=	good manufacturing practice
H_2O_2	=	hydrogen peroxide
hGH	=	human growth hormone
hGM-CSF	=	human granulocyte macro phage-colony stimulating factor
HMGB1	=	high mobility group box 1
HPV	=	human papilloma virus
HSP	=	heat shock protein
HSV	=	herpes simplex virus
ICD	=	immunogenic cell death
ID	=	intradermal
iDC	=	immature DC
IDO	=	indoleamine 2,3-dioxygenase
IFN	=	interferon
Ig	=	immunoglobulin
IL	=	interleukin
IM	=	intramuscular
IP	=	intraperitoneal
IP-10	=	inducible protein 10
IT	=	intratumoral
IV	=	intravenous
KIR	=	killer inhibitory receptor
LAG	=	lymphocyte activation gene 3
LN	=	lymph node
MAC	=	membrane attack complex
M-CSF	=	monocyte-colony stimulating factor

mDC	=	mature DC
MDSC	=	myeloid derived suppressor cell
MHC	=	major histocompatibility complex
MICA	=	MHC class I-related chain A
MIG	=	monokine induced by gamma IFN
MMP	=	metalloproteinase
MST	=	median survival time
MTD	=	maximum tolerated dose
MTX	=	mitoxantrone
MVD	=	microvessel density
NK	=	natural killer
NKG2D	=	NK group 2 D
NO	=	nitric oxide
NSAID	=	non-steroidal anti inflammatory drug
OD	=	optical density
OS	=	overall survival
PBMC	=	peripheral blood mononuclear cells
PBS	=	phosphate buffered saline
PD-1	=	programmed cell death-1
PDGFR	=	platelet-derived growth factor receptor
PDL-1	=	programmed death ligand-1
pDNA	=	plasmid DNA
PE	=	peak enhancement
PEG	=	polyethylene glycol
PgE2	=	prostaglandin E2
PMN	=	polymorphonuclear
Poly I:C	=	polyinosinic:polycytidylic acid
PR	=	partial response
PRRs	=	pattern recoginition receptors
Reg3b	=	regenerating islet-derived 3 beta
rIL-12	=	recombinant IL-12
SAP	=	serum alkaline phosphatase
SC	=	subcutaneous
SCC	=	squamous cell carcinoma
SD	=	stable disease
SFM	=	serum-free medium
shRNA	=	short hairpin RNA
STAT	=	signal transducer and activator of transcription
TAM	=	tumor-associated macrophage
TCR	=	T cell receptor
TDLN	=	tumor-draining LN
TERT	=	telomerase reverse transcriptase
TGF-β	=	transforming growth factor beta
Th1	=	T helper 1
TLR	=	toll-like receptor
Tmem	=	memory T cell
TNFR	=	tumor necrosis factor receptor
TRAIL	=	TNF-related apoptosis inducing ligand
Tregs	=	regulatory T cells
TSP-1	=	thrombospondin-1
TTP	=	time to peak
TVT	=	transmissible venereal tumor
uPa	=	urokinase-like plasminogen activator
US	=	ultrasound
UTR	=	untranslated region
VEGF	=	vascular endothelial growth factor
WiAU	=	wash-in area under the curve
WiR	=	wash-in rate

GENERAL INTRODUCTION

This chapter is partially based on

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One in three men and one in four women will develop cancer prior to 75 years of age,[1] as will one in every four dogs during its lifetime.[2] It goes without saying that cancer is an important cause of death. Indeed, cancer kills 31.5% of men and 23.6% of women in Flanders,[1] whereas nearly half of pet dogs over the age of 10 die from the consequences of cancer.[2]

Not only does cancer have a great social and emotional impact on patients and their environment, it also has a substantial financial impact on society. In 2006, the Belgian Health Fund reimbursed a total of 336 million euro for cancer, which is without doubt a considerable underestimation of the actual cost.[1]

Surgery, chemo- and radiotherapy are the current standard cancer treatment options in human and canine patients.[3] Yet often a small amount of cancer cells persists after therapy, causing relapse. This instigated the search for treatment options that complement standard therapies. Several alternative treatments have seen the light of day, e.g. hormone or endocrine therapy,[1] hyperthermia, photodynamic therapy, targeted therapy and immunotherapy.[4] Of these new avenues of research, especially immunotherapy has shown to be very promising in prevention of cancer recurrence.

Antitumoral immunotherapy is a therapeutic treatment option that stimulates the immune system of the cancer-bearing patient against the patient's cancer cells. Successes in immunotherapy for humans include among others increase in survival demonstrated by sipuleucel-T for prostate cancer,[5] ipilimumab for melanoma[6] and regression of high-grade premalignant lesions of the vulva through the therapeutic HPV16-vaccine.[7] Cancer immunotherapy has also been successfully applied in veterinary medicine. For cancer-bearing dogs, novel immunotherapeutics such as a melanoma vaccine (Oncept®) and a monocloncal antibody against canine B-cell lymphoma (AT-004) and T-cell lymphoma (AT-005) were developed.

1 Cancer animal models in the search for novel therapeutic alternatives

Mouse cancer models are a standard research tool for cancer in human cancer patients and offer a well-known platform to perform basic tests to evaluate the toxicity, potency and therapeutic efficacy of antitumoral drugs.[8] Their popularity is due to the various facilities available for research in mice. For example, many research tools are accessible for mice; the inbred mouse strains offer high standardization of experiment design as well as the ability to grow tumors from cancer cells derived from mice of the same strain. Furthermore, mice are easy to house as they do not require much space, food or maintenance. However, the translation of cancer research from mouse to man is far from optimal. Only one-third of highly cited animal research enters into clinical trials.[9, 10] Despite successful pre-clinical testing in rodent models, 85% of early clinical trials for novel drugs fail. The largest proportion of these failures occurs in trials for cancer drugs.[11]

It was postulated that most of the cancer vaccine trials in humans have failed due to elevated levels of circulating immunosuppressive cytokines and various immunological checkpoints in humans that may not be present in rodents.[12] Furthermore, it is true that human and mouse immune systems differ in the properties of both innate and adaptive immunity. These include discrepancies in leukocyte subsets, Toll-like receptors, natural killer (NK) cell inhibitory and activating receptors, Fc-receptors (FcR), immunoglobulin (Ig) subsets, some B cell and T cell signaling pathway components, γδ T cells, cytokines and cytokine receptors, Th1/Th2 differentiation, stimulatory molecule expression and function, antigen-presenting function of endothelial cells as well as in chemokine and chemokine receptor expression.[13] Moreover, in most of the rodent models, tumors are either artificially induced through injection of chemicals or through inoculation of a great number of cultured cancer cells, whereas tumor formation in humans is a long and complex process. Inbred rodents are not fully immune competent and therefore the complexity of spontaneously arising tumors in humans cannot be approximated with these models. Indeed, due to inherent differences in cancer development between rodent cancer models and human patients, rodent

models fail to faithfully mirror the extremely complex process of human carcinogenesis.[10] Consequently, rodent models result in a very low predictive power for antitumoral treatment efficacy in humans. [14, 15, 16] This leads to poorly efficient and expensive human phase I trials, prompting the need for an alternative intermediate animal model for human oncology patients. It is therefore recommended that therapeutic agents should be further evaluated in higher animal species after initial evaluation and positive results in rodents.[8] In addition, experiments should be designed in both genders and in different age groups of animals.[17] Since 1976,[18] diverse clinical trials have been conducted in dogs with spontaneous cancer and complemented the information obtained from more traditional research tools as murine cancer models and human clinical trials. Canine clinical trials have enabled researchers to collect information that would otherwise have been difficult or impossible to gather in either mice or humans alone.[19] There are obvious biological, practical and regulatory arguments for the use of spontaneous tumors in pet dogs as intermediate model in human cancer research.

From a biological point of view, canine clinical trials offer many advantages for human cancer research. Not only do dogs develop tumors in a syngeneic environment and in the presence of an intact immune system, they also share a similar histologic, biologic, and genetic cancer background significantly closer than the relationship between mouse and man.[20] In more detail, since cancer in dogs occurs naturally, a similar development and interaction between tumor, host and tumor microenvironment as in humans is evident.[19] For example, the spectrum of canine cancer is as diverse as in human patients and its initiation and progression are influenced by similar factors including age, nutrition, sex, reproductive status and environmental exposures.[21, 22, 23] Histological analogy was found to be present in melanoma, non-Hodgkin lymphoma, leukaemia, osteosarcoma, soft tissue sarcomas, and prostate, mammary, lung, head and neck, and bladder carcinomas.[19] Moreover, the intratumoral (cell-to-cell) heterogeneity in these cancers result in the same deadly features of human cancers (including acquired resistance to therapy, recurrence and metastasis).[19] Likewise, responses to treatment in dogs are analogous to men.[19] Furthermore, genetic molecular alterations

that drive cancers in dogs and humans are nearly identical.[20, 19] This is especially true for lymphoma, osteosarcoma, melanoma, glioma and soft tissue sarcoma.[19, 24, 25] These findings were possible through advances in technology and the release of a high-quality sequence covering 99% of the canine genome enabling the application of the same high-throughput methodologies in dogs that are used to examine human cancer.[20]
Although it has been argued that dog populations are more inbred than men, it has been demonstrated that the genetic diversity in both populations for a certain tumor type are similar.[14]

From a practical point of view, similar diagnosis and treatment options are available for dogs and humans. Indeed, adequate body size allows surgical interventions, medical imaging and tissue/blood sampling much like in human patients.[15], [24] The scientific climate today is very favorable for canine clinical trials with rapidly increasing species-specific research tools[15,, 24, 26] and veterinary infrastructure that meets the standards of good clinical practice.[26]
Trials in pet dogs also prove to be time-efficient. Some tumor types (e.g. osteosarcoma and brain tumors) occur much more in dogs than in humans. The higher the occurrence of certain tumor types, the more patients with these tumor types can be recruited for clinical trials and the faster this will lead to results. Furthermore, progression of cancer in companion animals is, on the one hand, slow enough to be able to compare responses to therapies and, on the other hand, fast enough to obtain results within a reasonable period of time.[15], [24] Since overall survival is the golden standard in evaluation of immunotherapeutic trials, the compressed course of cancer progression in pet dogs allows an effective and timely assessment of new cancer therapies.[19, 27] Indeed, whereas the assessment of disease-free interval or survival in human clinical trials usually takes several years, getting similar information from clinical trials in dogs generally takes much less time, just a few months in some cases.[28] These factors increase the feasibility of conducting clinical trials with sufficient statistical power.[24, 25] Moreover, the associated costs are lower than in human clinical trials.[24, 27]

The advantages of trials in pet dogs are not limited to cancer-bearing human patients. For the time being, the treatment options in veterinary oncology are limited. Pet dog owners seek the best possible care and are open to participation in clinical trials. Their contribution to the benefit of veterinary and human medicine is generally considered as positive. For these reasons, compliance with treatment, control visits and autopsy are exceptional.[24]

From a regulatory point of view, pet dogs have a broader access to clinical trials than humans. In human medicine, access to clinical trials is restricted to those refractory to standard treatment options. In veterinary medicine, this is not the case and any patient can participate as long as this is based on an informed owner's consent, approved by an ethical and deontological committee. This allows for evaluation of new therapeutic approaches in early stages of disease and in the setting of minimal residual disease after, for example, surgery or chemotherapy.[24] Research translation can be facilitated through collaboration between veterinary and human medicine and thus expensive phase I trials in humans optimized or even avoided. As a result, the present scientific and public climate is very favorable for implementing clinical trials with dogs as treatment models for humans. Close collaboration between veterinary and human clinical trials has led in the past to the development of a vaccine comprising of an adenoviral vector targeted to dendritic cells (DCs),[29] novel limb-sparing techniques in patients with osteosarcoma,[19] inhaled cytokine immunotherapy approaches in treatment of human patients with pulmonary metastases[19] and a DNA vaccine for melanoma in dogs (Oncept®), whose efficacy and safety led to ongoing clinical trials in patients with melanoma.[28]
Not only can pet dogs with spontaneous tumors aid in the development of new cancer therapeutics, they can also contribute to the study of environmental risk factors, as well as cancer biology, cancer progression and identification of cancer-associated genes.[19]

Future clinical studies in pet dogs are facilitated thanks to the completion of the canine genome, development of reagents for immunologic tests and flow cytometry and consortia such as LUPA (a European initiative taking advantage of the canine genome architecture for unravelling complex disorders in both human and dog), the US

National Cancer Institute's Center for Cancer Research's Comparative Oncology Program,[30] Zoobiquity's integrated and interdisciplinary approach to physical and behavioral health[31] and the Comparative Oncology Trials Consortium.[32]

2 Interactions between the immune system and cancer

A link between the immune system and cancer was observed by William Coley in 1890. He observed a significant reduction in tumor growth in cancer-bearing people diagnosed with erysipelas, a bacterial skin infection. Intrigued by this finding, Coley initiated the first immunotherapy trial in cancer-bearing patients by injecting live bacteria into the patient's tumor. Although a reduction in tumor size was apparent, fatal sepsis occurred in a number of patients.[33] Meanwhile chemo- and radiotherapy were introduced and the concept that the immune system has powerful antitumoral properties was unfortunately neglected for 80 years. In 1970, Bacillus Calmette-Guerin (BCG), an attenuated bacterium, proved to be an efficacious treatment option for bladder cancer.[34] In 1990, this treatment received an approval from the Food and Drug Administration (FDA) as a first-line therapy for superficial bladder cancer and it is still used today for the same indications.[35] Due to the narrow application possibilities of BCG-treatment (mainly suitable for superficial tumor types such as superficial bladder cancer), the research in cancer immunotherapy further concentrated on target-based therapy, may it be tumor-associated antigens or checkpoint inhibitors. This has led to FDA-approved immunotherapeutics such as Sipuleucel-T®, a dendritic cell-based cancer vaccine and Aldesleukin®, an interleukin-2 therapy for renal cancer.[36]

It is clear that the immune system is very closely involved in cancer development and plays a dual role. On the one hand, it can suppress tumor growth by killing cancer cells or inhibiting further expansion. On the other hand, it provides selective pressure by killing the cancer cells that are sensitive to the antitumoral immune response, thereby selecting for immune-resistant cancer cells. In addition, the immune system can facilitate conditions within the tumor microenvironment favorable for tumor outgrowth.[37] Whereas foreign organisms such as viruses and bacteria are easily

recognized by the immune system as foreign and induce a quick immune response, this is not the case for cancer cells, as they greatly resemble normal body cells. Thus, cancer cells can profit from this similarity to multiply as the immune system has no incentive for killing its own body cells. Since cancer cells greatly resemble normal body cells at the start of their development, which do not initiate an immune response due to self-tolerance, the tumor is initially invisible to the immune system. Yet, the more the cancer cells evolve into malignant cells, the more tumor-associated antigens are present and the more they become recognizable to the immune system.[37] Unfortunately, as cancer cells evolve, they also acquire immunoevasive mechanisms. Some of these mechanisms include hiding antigens necessary to initiate an immune response or secretion or presentation of immunosuppressive proteins that will neutralize the antitumoral efforts of the immune system. As a result, an uninhibited growth of these malignant cells ensues. The gradual interaction between the immune system and cancer cells is hypothesized to occur in three phases: immunosurveillance, immunoediting and immunoescape.[37]

2.1 Immunosurveillance

The ability of the immune system to discriminate between cancer cells and normal cells is referred to as tumor immunosurveillance.[37, 38] Indeed, cancer cells express antigens that differentiate them from their nontransformed counterparts, leading to their elimination by both the innate and the adaptive immune system.[37] Initially, in the stage of immunosurveillance, the immune system will efficiently recognize and kill aberrant cells.

The existence of immunosurveillance was first demonstrated when mice, immunized with chemically induced tumors, were protected against a subsequent rechallenge with the same tumor.[39] Nowadays this finding is supported by strong evidence, in both animal cancer and in human's.[37, 38] Chen and colleagues[40] proposed a step-by-step cycle of the generation of immunity to cancer. This whole step-by-step cycle can be divided into seven major steps: (1) release of antigens from the cancer cell, (2) cancer antigen presentation, (3) priming and activation of T cells, (4) trafficking of T cells to tumors,

(5) infiltration of T cells into tumors, (6) recognition of cancer cells by T cells and (7) killing of cancer cells.[40] Normally, a legitimate stimulus for the immune system is self-propagating at each step of the step-by-step cycle and leads to a broad T cell response.

2.2 Immunoediting

Swann and colleagues[41] hypothesized that, if the initial elimination of cancer cells by host immunity is not complete, cancer cells accumulate different genomic mutations and become more immune-resistant. Tumoral variants are selected after the cancer cells that were easily recognizable to the immune system have been eliminated. Such variant cancer cells are able to avoid or suppress the immunogical response and constitute the edited cancer.[38] Although host immunity cannot completely control these variant cancer cells, the adaptive immune system will have enough influence on the cancer cells to achieve a steady state situation. Thus, a type of tumor dormancy is evoked during the immunoediting phase.[37] However, for each step of the step-by-step cycle of the generation of immunity to cancer, cancer cells or tumor microenvironmental factors can develop inhibitory factors. These inhibitory factors are able to halt the development or limit the antitumoral immune response and lead to tumor escape.[40]

2.3 Immunoescape

Through continuous adaptation of cancer cells in response to the immune system or due to immune system deterioration, cancer cells become invisible to the immune system and are able to escape its control. These events finally tip the balance in favor of tumor growth and spread. In more detail, immunoevasion is mainly accomplished through lack of antigen presentation and creation of an immunosuppressive microenvironment (through production of immunosuppressive cytokines or recruitment of immunosuppressive regulatory immune cells).[42] Tumors can thus create a tolerogenic environment which spreads to draining lymph nodes (LN) and can enhance regulatory T cell (Treg) activity. These immunoevasive mechanisms lead to the

clonal expansion of less immunogenic cancer cells (by loss of tumor antigen) and prevention of apoptosis of cancer cells.[43]

3 Arms of the antitumor immune response

Cancer can be targeted through various arms of the immune system. In the design of an antitumoral immunotherapeutic treatment, it is important to target specifically rather than induce overall non-specific activation of the immune system.[40] However, it should be borne in mind that immunity results from a complex interplay between the innate (which is antigen-nonspecific) and the adaptive immune system (which is antigen-specific).[44] Furthermore, the antitumoral immune response can be abrogated by the immunosuppressive environment of the tumor. Therefore, the immunosuppressive tumor microenvironment should be tackled to allow for an effective antitumor response.[45]

The three principal arms of the immune system; the innate immune system, the adaptive immune system and the immunosuppressive arm of the immune system will be discussed in general. Then, immunotherapeutic targets for each arm will be reviewed as well as their application in canine clinical trials.

3.1 The adaptive arm of the antitumoral immune response

When the immune system is exposed to certain pathogens, it develops an immune protection against them. It is well-known that foreign entities such as viral or bacterial material are easily recognized by the immune system, resulting in their eradication. Alike, rodent models immunized with irradiated cancer cells were found to be able to resist tumor challenge when challenged with live cancer cells. This discovery led to the finding that cancer cells can also express antigens unknown to the host, that can be recognized by the immune system.[46] However, since cancer cells can also overexpress self-antigens, antitumoral immunotherapy delicately balances between non-self recognition and prevention of autoimmunity.[40] In general, the patient must be immunosufficient to elicit an effective antitumoral immune response.[47]

3.1.1 The general principle of adaptive antitumoral immune responses

The first step in the adaptive antitumor cellular immune response is the recognition and capture of tumor antigens by immature DCs (iDCs). The DCs subsequently degrade and cleave the tumor antigens into peptides, which are processed into the MHC class I or II pathways to be (cross-) presented on their cell surface. When a damage-associated molecular pattern (DAMP) or a pathogen-associated molecular pattern is present, the iDCs undergo maturation. This process is characterized by an increased secretion of cytokines, an increased expression of costimulatory molecules CD80 and CD86, as well as chemokine receptor 7 by the DCs, which induce their migration into the LN. When the antigen-loaded DC arrives in the LN, it undergoes an additional activation step called 'licensing', mediated either by direct interaction of CD40 on the DC with the CD40 ligand on the CD4+ T cell or by cytokines.[48] Both CD4+ and CD8+ T cells require a minimal number of MHC–peptide T-cell receptor (TCR) interactions with the mature DCs (mDCs) to become activated[49] and for optimal activation, interactions between additional ligands on the mDCs (4-1BBL, OX40L, ICOSL) and the respective receptors on the CD4+ and CD8+ T cells (4-1BB, OX40, ICOS) are required.

The activation of CD4+ T cells is essential for many more features of the adaptive immunity besides activating and maintaining CD8+ T cells.[50, 51, 52] Lack of properly activated DCs in the absence of CD4+ T cells can induce tolerance rather than activation of CD8+ T cells.[53] Activated CD4+ T cells can develop into Th1 or Th2 cells, of which the former will produce cytokines to activate a Th1 response and the latter a Th2 response (generally characterized by a mainly pro-inflammatory and cellular immune response versus an anti-inflammatory and humoral response, respectively). CD4+ T cells also assist in the infiltration of CD8+ T cells in the tumoral mass. They recruit macrophages, granulocytes and NK cells to directly kill cancer cells and enhance angiogenesis. CD4+ T cells are additionally fundamental for the induction of long-term memory CD8+ T cells.[54] Therefore, CD4+ T cells determine the nature of the immune response.[49, 52, 55, 56]

The CD8+ T cell is crucial in the cellular immune response, particularly the CD8+ cytotoxic T lymphocyte (CTL).[44] They provide the strongest antitumor immune

response by directly killing cancer cells that present peptide–MHC class I complexes. Interaction of CD80 or CD86 on the DC with CD28 on the CD8+ T cell causes activation of the CD8+ T cells, which results in IL-2 release and proliferation of T cells (*Figure 1*).

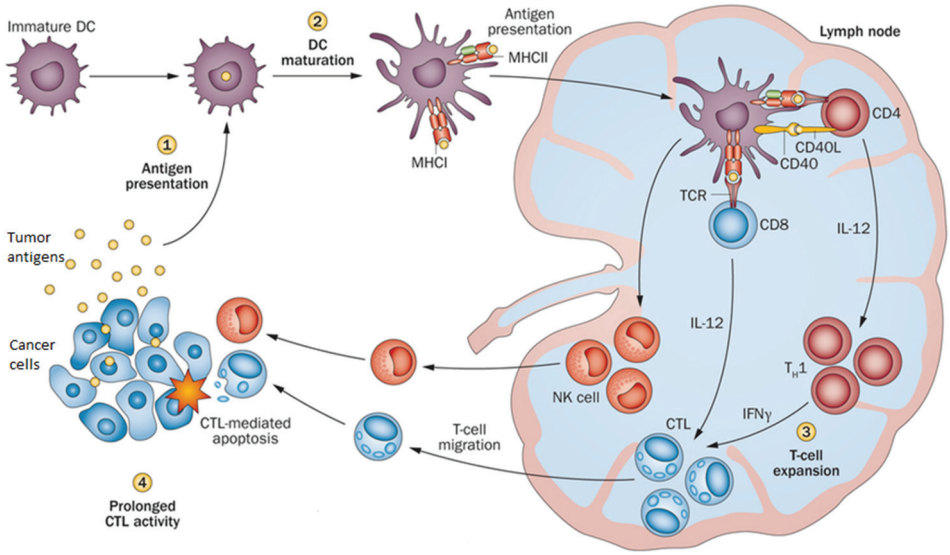

FIGURE 1. Principle of the adaptive immune response. Adapted from Melero 2014 [57]

DCs are not only critical for cellular responses but also have an important role in controlling humoral immunity. They do so both directly by interacting with B cells and indirectly by inducing the expansion and differentiation of CD4+ helper T cells.[44] The humoral immune response is characterized by the production of antibodies by B lymphocytes. Antibodies bind onto the surface of a foreign antigen, marking it for other cells of the immune system. For instance, when NK cells recognize antibody-coated cells, they will destroy these cells through antibody-dependent cell-mediated cytotoxicity (ADCC).[58] Furthermore, the complement system consists of a collection of proteins capable of binding antibodies, whereafter these proteins initiate a proteolytic cascade. This cascade results in lysis of the antibody-bound cell. Moreover, antibodies are capable of blocking signal transfer of for instance growth factors.[58] In turn, the lysis of cancer cells through ADCC and/or the complement system induces cancer cell

fragments and thus the release of tumor antigens, which can be presented by antigen-presenting cells (APCs). B cells themselves can function as an APC and when activated, B cells produce cytokines that direct the differentiation of naive CD4+ T cells into effector CD4+ T cells.[58]

3.1.2 Antitumoral application of the adaptive arm of the immune system via cancer vaccines

The adaptive immune system is able to eliminate cancer cells. Cancer cells express new foreign antigens and/or overexpress self-antigens while the adaptive immune system is able to specifically target cancer cells. Considering the immune system can be taught to specifically recognize and eliminate foreign material through vaccination, cancer antigens can be assembled into a vaccine and used to immunize the patient against its own cancer cells. Most cancer vaccines so far are used as (experimental) therapeutic vaccines, although some are deployed prophylactically (e.g. HPV-vaccine Gardasil®). In many phase I/II studies, cancer vaccines have shown clinical benefit in all species, in particular extended overall or disease-free survival. In contrast, objective durable regressions of the type associated with targeted or immunomodulatory monoclonal antibody therapy or chimeric antigen receptor (CAR) adoptive T cell therapy were rarely seen.[59] Although both active (cancer vaccines) and passive (adoptive T cell transfer) approaches have the ability to be extremely specific for an antigen of interest, one important difference is the inability of passive approaches to confer memory.[60] Cancer vaccines aim to reeducate the immune system to create an immune memory in order to prevent cancer recurrence.[61, 62, 63, 64] In addition, cancer vaccines are currently the most efficient and cost-effective means of generating tumor specific immunity.[65]

3.1.2.1 Cancer vaccine types

All cancer vaccines deliver tumor antigens, but the form of the antigen can vary according to the cancer vaccine type. Antigens can be provided through (whole) cancer cell vaccines, genetic vaccines and peptide or protein vaccines.[66] Whole cancer cell vaccines possess several advantages over the other cancer vaccine types. When cancer

cells are administered, all relevant antigens to evoke an antitumoral immune response are present. There is no need, in contrast to genetic vaccines or peptide and protein vaccines, to identify tumor antigens or to select immunodominant epitopes on the tumor antigens. The antigens present on the cancer cells in whole cancer cell vaccines are varied and evoke an MHC independent broad-ranged anticancer response. Furthermore, both CD8+ and CD4+ T cells will be stimulated, as the antigens provided by whole cancer cell vaccines contain epitopes for presentation to both cell types. This delivery method thus generates a varied and broad immune cell activation which greatly diminishes the chance of tumor escape. In contrast, non-mutated self-antigens have often been selected for peptide/protein or genetic vaccines. However, when self-antigens are not mutated, they are likely to induce a less efficient antitumoral immune response. Indeed, there is a small chance that they will generate high-avidity clones and if memory T cells (Tmem) exist toward them, they will often include Tregs.[44] Autologous cancer cells will contain both non-mutated and unique mutated self-antigens, thus maximizing the chance of immune recognition.[66] In contrast, peptide and protein vaccines are more limited in their generation of broad antitumoral immune responses as their response is MHC-restricted and limited to the selected epitopes.[66] For genetic vaccines, the antigens will mainly be channeled into the MHC class I presentation pathway, resulting in a less broad immune activation than whole cancer cell vaccines.[67] However, recently DNA vaccines have been constructed that produce antigens that are secreted, thus being able to access the MHC II pathway.[68] Whole cancer cell vaccines showed a higher objective clinical response rate than peptide, protein or genetic vaccines.[66, 69, 70, 71]

3.1.2.1.1 Peptide and protein cancer vaccines

The successful induction of a T cell response requires stable presentation of T cell epitopes on MHC complexes. The antigen of interest can be introduced by vaccination with the whole protein, or with short or long synthetic peptides derived from this protein.[72]

Cancer cells express peptide antigens recognized by CD8+ CTLs, which are typically 8-10 amino acids long and are presented in association with MHC I. The peptides

recognized by CD4+ T cells are presented in association with MHC II and are usually longer (13-18 amino acids in length).[73] Peptide vaccines have several benefits, including easy synthesis and administration, target specificity, convenient storage, their safety (as demonstrated in many trials) and effective induction of T cell responses.[73] Although MHC-restriction of individual peptides limits their use to a subset of patients, it was observed that mixtures of a dozen of differently MHC-restricted peptides can induce immune responses in the 85% of patients with melanoma who express one or more of those MHC molecules.[73] An increase of the antigen dosage directly correlated with immunological response rates and frequency of circulating, peptide-specific CD8+ T cells; however, plateau responses have been observed.[72] Since most short peptide vaccines are injected in non-physiologically high amounts, they are likely to clog up the appropriate MHC class I molecules of non-professional APCs in the absence of costimulatory molecules.[59] This interaction can result in tolerance toward the bound peptides.[73, 59] Moreover, these short peptides have little or no tertiary structure and thus are subject to rapid degradation by tissue and serum peptidases. Furthermore, the stability of the peptide-MHC interaction on the DC is probably lower than the stability of processed whole protein and thus less likely immunogenic.[73] Indeed, peptide cancer vaccines resulted in overall clinical response rates of only about 2.9%.[62] Thus, these vaccines are not yet optimized and require more research on antigen and adjuvant improvement.[73]

Whole tumor proteins are uptaken and processed by APCs into MHC-associated peptides. The advantage of vaccination with whole tumor proteins is not only that they could provide peptides for binding to a large array of MHC molecules (therefore available to virtually all patients) but also that no prior knowledge of T cell epitopes is required.[72] However, a shortcoming with the use of protein-based therapeutic vaccines is that they mainly induce responses to dominant epitopes, resulting in a rather narrow T cell response.[72]

A compromise between the advantages of peptide and protein vaccines is the use of longer peptides. Various studies suggested that longer peptides induce better CTL responses, presumably because they are more easily endocytosed, processed and presented by APCs than whole proteins.[72] The advantage of using long synthetic

peptides is that they have the capacity to induce immune responses to subdominant epitopes, which are not induced when vaccinating with short peptides (covering exact epitopes) or with proteins. Indeed, T cells specific to subdominant epitopes also contribute in anti-tumor immune responses. Therefore, the broadest variety of T cell specificities and the best anti-tumor immunity is probably generated when vaccinating with a pool of long synthetic peptides covering the entire protein sequence.[72]

3.1.2.1.2 Genetic vaccines

Genetic vaccines utilize DNA (as plasmids (pDNA)) or RNA (as messenger RNA (mRNA)) to deliver an expression cassette carrying the coding region of the antigen(s) of choice *in vivo*. Cells that have uptaken the pDNA or mRNA translate the constructs into protein, which results in the production of the selected antigen.[2, 74] Transfection can be mediated through simple injection of "naked" pDNA or mRNA, or its delivery enhanced through gene-gun delivery, electroporation or association with liposomes or other particles.[74, 75]

DNA vaccines are bacterial plasmids constructed to express an encoded protein following *in vivo* administration and subsequent transfection of cells.[75] DNA delivery vehicles contain a gene expression cassette usually controlled by a strong constitutive promoter. DNA inoculation must be followed by cell uptake and localization inside the cell nucleus to give rise to a transcriptionally active form.[2] DNA vaccination elicits cell- and antibody-mediated responses to a variety of antigens. The amount of proteins expressed, its persistence and the kind of antigen presentation are key variables that determine the efficacy of the protection thus elicited.[76] Overall, various studies have shown DNA vaccines to be safe, with fever and pain, redness, and swelling at the injection sites the most common adverse events reported.[77]

Besides DNA, the naturally transient and cytosolically active mRNA is seen as a possibly safer and more potent alternative to DNA for gene vaccination.[75] Similar to DNA vaccines, they are constructed to express an encoded protein following *in vivo* administration and subsequent transfection of cells.[75] Self-replicating mRNA vaccines are potentially more potent than DNA vaccines because double-string (ds)RNA is

formed during mRNA replication which is very immunogenic as it resembles viral dsRNA.[78]

Many advantages have been attributed to RNA vaccines compared to DNA vaccines. RNA vaccines elicit a transient effect, require only cytoplasmic expression (DNA needs to cross the cell and nucleus membrane to be functional) and there is no risk for genome integration. Yet the above-listed advantages are not black and white and mostly theoretical. DNA vaccines do not induce a life-long expression. Their effect is mostly transient as well, due to cell replication, loss or inactivation of the plasmid.[79] Integration of pDNA into the genome following intramuscular injection is at least three orders of magnitude below the spontaneous mutation rate. This suggests that the risk for mutation due to integration of plasmid DNA vaccines is negligible after intramuscular injection.[80] Electroporation increases the risk for chromosomal integration, yet still at a lower rate than spontaneous mutations.[81] Furthermore, while mRNA only needs to cross one cell membrane to be functional, pDNA-mediated protein expression greatly surpasses mRNA-mediated expression after electroporation.[82] On the other hand, clear advantages of mRNA are that, unlike pDNA, RNA can be produced by a cell-free enzymatic transcription reaction, thus avoiding the use of micro-organisms or cultured cells in manufacturing, with associated quality and safety issues. This method enables simple downstream purification and very rapid and cost-effective manufacturing.[75]

Although mRNA is known to be less stable than pDNA vaccines, lyophilization studies have shown that RNA vaccines are not less stable than conventional vaccines that require a cold chain to be effective. Therefore, mRNA can be produced in large amounts and with good manufacturing practice (GMP) quality, thus allowing further development of mRNA-based therapies.[75]

As new cancer antigens come to the forefront with novel RNA encapsulation and targeting techniques, RNA vaccines may prove to be a vital, safe and robust method to initiate patient-specific anti-tumor efficacy.[78] Replicon mRNA (self-amplifying mRNA) possibly fills the gap between RNA and DNA vaccine structures as it combines a prolonged expression and a better transfection efficiency.[75]

3.1.2.1.3 Dendritic cell vaccines

DCs are rare leukocytes that are uniquely potent in their ability to present antigens to T cells and form a link between innate and adaptive immunity. These properties have prompted their application to therapeutic cancer vaccines.[44, 83] DC vaccines consist of DCs loaded with tumor-specific antigens. This can be achieved either by culturing *ex vivo* autologous DCs with an adjuvant that induces DC maturation and the tumor-specific antigen followed by injecting these cells back into the patient, or by inducing DCs to take up the tumor-specific antigen *in vivo*.[44] DCs that are generated *ex vivo* by culturing haematopoietic progenitor cells or monocytes with cytokine combinations have been tested as therapeutic vaccines in canine and human cancer patients for more than a decade. These studies concluded that DC-based vaccines are safe and can induce the expansion of circulating CD4+ T cells and CD8+ T cells that are specific for tumor antigens.[44, 83] However, apart from the results with sipuleucel-T (Provenge®), no successful phase III trial results have been reported for DC-based cancer vaccines.[59]

3.1.2.1.4 Whole cancer cell vaccines

The source and the characteristics of cancer cells will have an important impact on the efficacy of whole cancer cell vaccines. Additionally, whole cancer cell vaccines can be manufactured and administered in different ways, resulting in diverse effects on the immune response. In this section, we will discuss these aspects in more detail.

CANCER CELL SOURCE FOR WHOLE CANCER CELL VACCINES

For the preparation of whole cancer cell vaccines, cancer cells derived from cell lines or from primary tumors can be used.[84] Furthermore, cancer cells can be provided by the patient (autologous) or by a donor (allogeneic).[85]

In theory, primary cancer cells are preferred over tumor cell lines. The antigen spectrum of tumor cell lines can change after long-term culture[86, 87], whereas primary cancer cells are counterselected by the immune system and potentially provide additional tumor antigens.[87] However, in practice and depending on the tumor type, only a minority of primary cancer cells can be maintained in culture,[88] limiting their use in whole cancer cell vaccines. Cancer stem cells (CSCs) are considered superior to the

unselected cancer cells as an antigen source.[84, 89] It has been hypothesized that CSCs drive both local cancer recurrence and systemic relapse, which makes specific targeting of CSCs very attractive.[89] In experimental mice, CSC vaccines elicited humoral as well as cellular immune responses against CSCs[90] resulting in an efficient protective anticancer immunity.[89]

Autologous cancer cells are a better antigen source than allogeneic cells when only the raised anticancer immune response is taken into account.[91] The autologous vaccine will contain unique tumor antigens encoded by gene mutations specific to that individual tumor. These antigens might be more immunogenic than commonly shared tumor antigens and result in stimulating effective and long-lasting anticancer responses in the canine and human patient.[91] In contrast, the complexity of vaccine manufacturing for individual patients (such as the need for a sufficient amount of cancer cells, the collection of these cells, problems concerning standardization, quality control) and its usefulness limited to a single patient has led to the use of allogeneic cancer cells.[66] The development of cancer vaccines based on allogeneic cancer cells is possible through the use of cell lines, which are preferred over allogeneic primary cells as they harbor most of the same production complexities as autologous primary cells. These cell lines are selected to provide a limitless source of multiple tumor-specific antigens and a broad range of MHC expression.[56] Allogeneic vaccines can, however, only achieve clinical effectiveness if they adequately represent the characteristic tumor antigens of the patient's cancer cells.[70] Alas, allogeneic cancer cells will seldom contain the same tumor antigens as autologous tumors, even when combining multiple allogeneic cell lines.[85] The most efficient response will probably be provided by autologous cancer cells that express an allogeneic molecule. Allogeneic MHC molecules could hereby act as an adjuvant, without overwhelming the cancer-specific CTL response.[92] However, it should be borne in mind that adding allogeneic elements in vaccines will potentially decrease their efficacy after repeated vaccination through alloreaction, which destroys the vaccine before it even has the chance to generate the desired response.[93]

Effect of immunogenic cell death of cancer cells in cancer cell vaccines

When a cell dies, it can be cleared with (immunogenic) or without (nonimmunogenic) stimulating an immune response against its dead cell antigens.[47] In cancer cell vaccine

manufacture, immunogenic cell death (ICD) is the objective. An immunogenic death depends on the ICD-inducing stimulus.[47] Multiple ICD-inducing stimuli have been described and their main characteristic is the ability to induce expression and release of DAMPs from the cells they have killed. The DAMPs that are expressed and released by the cancer cells after ICD can interact with pattern recognition receptors on many immune cells according to a defined spatiotemporal pattern. Binding of DAMPs to pattern recognition receptors results in the release of cytokines and chemokines by the immune cell. As a result, APCs are stimulated to efficiently take up and process tumor antigens and cross-prime T cells[94] (*Figure 2*). Multiple factors can influence the efficacy of DAMPs.[87] The immunogenicity of the whole cancer cell vaccine is influenced by the death-inducing stimulus, the produced DAMPs themselves, the DAMP location, and the combination of cancer- and host-associated factors.[50, 95, 96, 97, 98] The cancer cell has to be able to express the DAMPs, which in turn should bind immune cells and not cancer cells.[87] Indeed, binding of DAMPs to toll-like receptors (TLRs) expressed on cancer cells can promote cell survival and chemoresistance.[87]

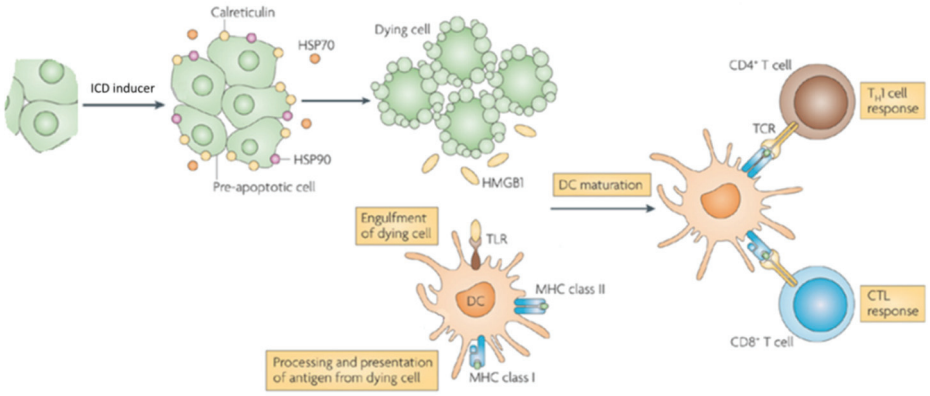

FIGURE 2. Principle of immunogenic cell death (ICD). Legend: CTL: Cytotoxic T cell, HMGB1: High Mobility Group Box 1, HSP70: Heat Shock Protein 70, MHC: Major Histocompatibilty, TLR: Toll-like Receptor. Adapted from Green 2009 [99].

Application of cancer cell vaccines

Antigen dosage of cancer cell vaccines

Despite observed tumor eradication in experimental animal studies, limited success has been obtained so far in clinical trials for whole cancer cell vaccines in humans.[100, 101] However, it is possible that the antigen dosage generally used in vaccine studies is inadequate to initiate an optimal anticancer response in humans. The amount of cancer cells, DCs or cancer cell–DC fusion cells generally used for the vaccination of humans was comparable to the amounts used in mice (mamma cancer mouse[102, 103, 104, 105, 106] / human[107, 108, 109,110]; melanoma mouse[111, 112, 113, 114, 115]/human[116, 117, 118]). Although the minimal antigen dose for antibody responses is independent of the body size,[119] this is not true for cellular responses.[120] T cells are highly dose-responsive and when the mice doses of antigen are used in humans, they are likely ineffective at raising T cell responses.[120] The marked difference in the biology of secondary lymphoid tissue between mice and humans adds to this inappropriate extrapolation of doses. The average volume of a human LN is larger and the local draining lymphoid tissue is far more diffuse than in the mouse. This dilution of antigen may result in a local concentration of antigen insufficient to initiate effective T cell priming.[120] The optimal quantity of the antigen in human whole cancer cell vaccines remains to be determined and undoubtedly depends on the antigen properties, the adjuvant and target species, and should ideally be determined for each antigen.[121]

Administration route of cancer cell vaccines

It is not clear which method of administration is most immunogenic for cancer vaccines.[71] DCs injected intravenously (IV) primarily accumulate in the lungs and subsequently redistribute to the liver, spleen and bone marrow in human cancer-bearing patients. Those injected intradermally (ID) or subcutaneously (SC) migrate to the regional LNs.[122] The content of an intraperitoneal (IP) injection is distributed to the mediastinal LNs and the liver.[123] Activated DCs can prime cellular immunity regardless of the route, yet the quality of this response and the induction of the humoral response may be affected by the route of administration.[123] Cancer type and location, timing of vaccination and location of injection relative to the tumor will also be related to the evoked responses.[71] In humans, it has been reported that ID administration is superior

to IV vaccination[55] or IP vaccination.[71] Although some researchers challenged the superiority of intranodal (IN) vaccination,[124] many researchers claimed IN vaccination to be superior to IV,[55, 125, 126] ID,[127] SC or IP vaccination.[125] However, IN injection of vaccines is difficult.[128] Technical problems might explain the observed inferiority of IN vaccination in the study of Engell-Noerregaard and colleagues.[124]

Location of vaccination relative to the tumor

The location of vaccination relative to the tumor has an important influence on the effectiveness of cancer vaccines.[71] Peritumoral vaccination will activate T cells centered in tumor-draining LNs (TDLNs), a location favorable for T cell trafficking into the tumor, and elicits anticancer effects more rapidly than distal vaccination.[129] However, TDLNs are often actively tolerogenic as these LNs contain locally secreted cytokines such as transforming growth factor (TGF) β, PgE2 and IL-10.[45] On top of that, they harbor immunosuppressive cells such as Tregs, iDCs, mDCs expressing PDL1 and indoleamine 2,3-dioxygenase (IDO). All of these elements are responsible for a potent immunosuppressive environment, leading to a tolerizing cross-presentation of tumor-derived antigens by the host cells present in the TDLN. The increased levels of Treg activity are localized to TDLNs and do not occur in the other LNs of the same host.[45] Subsequently, peritumoral vaccination is not preferred when the tumor has already established immunosuppressive features. This has been documented by Ohlfest and colleagues who observed in an experimental setup that immune responses decreased as the vaccine was administered closer to the tumor, whereas a significantly higher site-specific immune response was observed in tumor-free mice.[130] Furthermore, tumor antigen administration does not need to take the location of DC types in the body into consideration, as DC location is not a limiting factor in the generation of an effective anticancer immune response. Indeed, the study of Ali and colleagues demonstrates that it is possible to attract DCs which are typically localized to secondary lymphoid structures toward a subcutaneous vaccine site in mice.[131] These DCs also proved to be necessary for an efficient immune response, despite the fact that tissue-specialized DCs impart tissue-specific homing properties to CD8+ T cells.[71]

Administration schedule of cancer cell vaccines

The current consensus dictates weekly, biweekly or monthly boosters,[67, 71] yet daily exogenous antigen immunization with peptide/protein cancer vaccines for several consecutive days (socalled cluster immunization) has proven to be clearly superior to booster immunization with greater intervals.[132] When previously immunized mice were boosted with cluster immunization of peptide/protein cancer vaccines, it resulted in a CD8+ T cell peak response that surpassed the primary immunization level. This was not the case when a single second immunization was conducted after a single primary immunization.[132] Interestingly, an adjuvant is needed to obtain the superior effect of cluster immunization. Indeed, Wick and colleagues found that immunization of mice with four consecutive daily doses of exogenous antigen without an adjuvant failed to evoke any antigen-specific T cell response.[132] However, depending on the administered adjuvant, daily vaccination is not always possible as, for example, daily CpG exposure causes damage to secondary lymphoid organs and diminishes CD8+ T cell responses, while poly I:C does not.[133] There is little known about how the timing of whole cancer cell vaccine administration may change clinical efficacy. Daily vaccination is probably not needed when using cancer cell–DC fusion cancer vaccines, as the fusion cells will produce, process and present tumor antigens for several days after the fusion.[49]

Conclusion of application of whole cancer cell vaccines

Despite high expectations in whole cancer cell vaccination trials, thus far reported clinical responses have been rather disappointing. Many variables in the vaccine manufacture require optimization. Until today, the optimal dosage of the cancer vaccines remains to be elucidated; there is evidence that the current dose of antigens is too low for the treatment in humans. The therapeutic design should take the properties of immune response phases into account.[48] Ideally, first the specificities not only of the cancer but also of the patient should be identified.[134, 135] Afterward, tolerance should be suppressed, followed by the delivery of the adjuvanted cancer vaccine and accompanying therapies to enhance antigen presentation and to boost T cell priming. This treatment plan is ultimately completed with strategies that augment T cell efficacy and memory.[48]

3.1.2.2 Adjuvants in cancer vaccines

The immune response induced by cancer cell vaccines can be significantly enhanced by the use of the appropriate adjuvants.[136] There are effector adjuvants on one hand and memory-inducing adjuvants on the other hand. Eradication of cancer cells and the simultaneous buildup of immune memory are not easily generated by one single adjuvant type. IL-12 and type I interferon (IFN) are known to induce potent differentiation of T cells into effector cells.[120] Tumor necrosis factor receptor (TNFR) ligands (such as OX-40, 4-1BB and CD27) activate protein kinase B which directs T cell differentiation more toward Tmem than toward primary effector T cells.[120] The primary and secondary CD8+ T cell responses rely more on CD27 than OX40 for their generation and vice versa for the CD4+ T cell response.[120] Dosage is the key to success for effector as well as memory-inducing adjuvants.[120] An excess of effector adjuvants can drive responding cells into terminal differentiation and thus become a weak point for the generation of immune memory.[137, 138] Moreover, Burchill and colleagues also demonstrated that overstimulation of CD27 has a negative effect on the development of competent CD8+ T cell memory.[120] It is important that the dosage is tailored to the individual adjuvant.

3.1.2.2.1 Persistence of antigens by adjuvants that generate a depot effect

Antigens should be presented long enough to enable a robust immune response, yet short enough to enable an efficient memory response.[52, 131] In the search for an ideal vaccine, it was long assumed that an antigen depot is necessary to provide a sufficiently long antigen contact. However, although depots provide a longer presence of antigens and thus a longer possibility for the immune system to interact with these antigens, not all depots provide the aspired immune stimulating effect. It has been demonstrated that an antigen depot formation can trap T cells generated by the vaccine, and hence prevent them from reaching the desired target cancer site.[120, 139] Therefore, Hailemichael and colleagues suggested that cancer vaccines with a short-lived depot effect might result in enhanced therapeutic efficacy.[139] In some adjuvants an unwanted depot effect is created by their formulation, such as those based on saponin, MF59, Montanide ISA 51 or QS21, which require an emulsion for the formulation of the adjuvant. Adjuvants

such as the TLR-based adjuvants poly I:C, monophosphoryllipid or CpG do not require an emulsion formulation[120] and are therefore suitable for cancer vaccination.

3.1.2.2.2 Timing of adjuvant administration

An efficient and durable T cell response depends on signals that are either received in the first few days of antigen recognition by naive T cells or later, when memory cells come across this antigen once more.[140] TNF/TNFR family members are induced hours to days after TCR affiliation.[134] Several adjuvant vaccination schemes might sufficiently skew the anticancer immune response in the desired direction. One option is alternating priming for several consecutive days with an effector and a memory adjuvant, followed by a boost with a memory adjuvant. Alternatively, priming with an effector adjuvant only, followed by a well-timed secondary boost with a memory adjuvant. It is important to administer the memory adjuvant when the memory cells start to develop and require maintenance through sustained protein kinase B activation.

3.1.2.3 Limitations in cancer vaccines

3.1.2.3.1 Genetic defects in cancer cells and patients

The efficacy of a cancer vaccine treatment depends on the possibility of the patient and/or the patient's cancer to respond to the vaccine. Certain genetic defects in patients or tumors may compromise the efficacy of cancer vaccines or adjuvants.[141, 142] For example, it has been demonstrated that TLR4 is essential for efficient tumor antigen cross-presentation.[143] However, 12% of Caucasians are TLR4 deficient which may imply an inefficient cross-presentation of tumor antigen in these patients.[143] Compensatory treatments have been developed to specifically tackle TLR4 deficiency.[47] It is therefore important to assess the immunogenic characteristics of cancer patients and adapt the vaccine manufacture where needed. Furthermore, tumor immunogenicity varies greatly between different types of cancer and even between cancers of the same type (in different individuals).[144] A cancer type can differ in specific transduction pathways[95, 97] or in the expression of endogenous cytokines or the amount of MHC class I molecules.[100] Additionally, certain tumors can expose (e.g., CRT) or release

immunogenic signals (e.g., ATP and HMGB1) in response to a death-inducing stimulus, whereas others do not.[95, 97] These deficiencies can be compensated for via the addition of, for example, recombinant CRT, ATP agonists and TLR4 agonists to cancer cells that do not express CRT or release ATP and HMGB1, respectively.[143] The development of a scanning assay for missing ICD pathway factors in a cancer patient is the future to optimized patient-specific cancer cell vaccines. Nowadays, not all relevant missing factors are known yet[47] and running an elaborate checklist for each possible missing factor in every single cancer patient would further increase the vaccine production costs.

3.1.2.3.2 Heterogeneity in patient selection and tumor size

The large variety in reported clinical success of DC-based cancer vaccines in humans might be partially explained by the selection of the enrolled cancer patients. Most eligible are patients with early-stage cancer and patients who had a successful first-line therapy and had minimal prior chemotherapy. Moreover, the tumor is preferably not very aggressive in order to allow the patient enough time to receive multiple rounds of vaccination.[145] In current trials, often advanced-stage patients with high tumor burdens and/or patients who need more time than granted to respond to the treatment are assessed.[145] Tumor size has a great impact on the efficiency of anticancer vaccines. Decreasing the tumor burden prior to vaccine treatment allows a higher cure rate since smaller tumors are more effectively eradicated than large ones.[129] Unfortunately, most current mice models provide a proof of concept for the immunogenicity of the vaccine in a rather prophylactic than therapeutic setting. In the majority of preclinical studies with mice, cancer vaccination is performed a few days after inoculation, not taking into account the impact that slowly progressing tumors have on the adaptive immune system.[63] In many studies, the cancer vaccine is given even before the inoculation of the cancer cells.[62]

3.1.2.4 Cancer vaccines in dogs (peptide, protein, genetic and cell vaccines)

Successful cancer vaccine results in companion animals can often be translated to human medicine.[16] Prior to clinical trials in cancer-bearing pet dogs, several cancer

vaccines were evaluated in healthy laboratory dogs. Three important conclusions were drawn from preclinical vaccination studies in healthy laboratory dogs. First, it was confirmed that cancer vaccines generate an antitumor immune response. Second, no autoimmune or other serious adverse effects were detected. Third, clinical efficacy of DC vaccines and DNA vaccines was demonstrated in dogs with transmissible venereal tumor (TVT) .[146]

Cancer vaccines in dogs have been applied for several tumor types and generated a range of treatment results (*Table 1, Table 2, Table 3*). Melanoma and lymphoma are the most popular tumor types studied so far. For melanoma, the best results were obtained in the study of Finocchiaro and colleagues.[147] Melanoma's were completely or partially excised and the tumor bed or tumor tissue respectively were co-injected with lipoplexes bearing Herpes simplex virus (HSV)-thymidine kinase and IFNβ genes and ganciclovir at the time of surgery. This approach was combined with the periodic administration (concurrent with the surgery) of a SC genetic vaccine composed by tumor extracts and lipoplexes carrying the genes of human IL-2 and human granulocyte-macrophage colony-stimulating factor. The control group consisted of patients in which melanoma was completely or partially excised. Overall survival of the group with complete excision and immunotherapy was 7 times higher than in the control group that had complete excision only (704 (99-2251) versus 101 (11-568) days). Overall survival of the group with partial excision and immunotherapy was 4 times higher than in the control group that had partial excision only (323 (46-1321) days versus 78 (29-206) days),[147] thus potentially indicating the impact of tumor load on immunotherapeutic approaches (*Table 1*).[147]

Also for lymphoma, a significant increase in overall survival was obtained with a cancer cell vaccine (unspecified lymphoma,[18] B cell lymphoma[148]) and a DNA vaccine (B cell lymphoma).[149] All studies had concurrent control groups that indicated a significant survival. Already in 1976, Benjamini and colleagues administered an IM autologous tumor cell lysate, admixed with Freund's complete adjuvant to 11 canine lymphoma patients in complete remission after chemotherapy. The control group consisted of patients having received chemotherapy and a placebo vaccine (vitamin B12) only (n=

9). A median survival time of over 348 days was obtained in the vaccinated group, which was significantly higher than the 197.5 days in the chemotherapy only group.[18] Thirty-eight years later, similar results were obtained by administrating an ID autologous tumor cell lysate, admixed with hydroxylapatite on top of chemotherapy in 12 lymphoma patients.[148] The control group (n= 7) received chemotherapy and a placebo only (the same amount of hydroxylapatite without the tumor proteins). A lymphoma-specific survival of 505 days was obtained in the vaccinated group, whereas only a median of 159 days was reached in the control group.[148] Gavazza and colleagues administered IM and subsequently electroporated a telomerase reverse transcriptase DNA vaccine to 21 dogs with lymphoma combined with chemotherapy, whereas the control group (n= 21) received chemotherapy only. A significant increase in overall survival ensued (532 days vs 205 days) (*Table 2*).[149]

Besides melanoma and lymphoma, other tumor types have been treated with cancer vaccines. For haemangiosarcoma, fibrosarcoma, osteosarcoma, mammary gland tumors, glioma, meningioma, astrocytoma or bronchoalveolar adenocarcinoma, no concurrent control groups were included, thus preventing sound conclusions (*Table 3*).

The absence of concurrent randomized control groups complicates comparisons between cancer vaccine trials conducted in cancer-bearing pet dogs. Many published trials use different treatment schedules, tumor antigens, delivery systems, patient selection criteria and a low patient number.

Boston and colleagues conducted a retrospective study in 151 dogs with melanoma and found no survival benefit with any systemic adjuvant therapy compared to surgery only.[150] Yet, the authors emphasize that this is possibly due to the low number of dogs in each treatment group in their study. Indeed, together with the inherent study limitations including lack of randomization and treatment protocol standardization, it was made impossible to detect any differences in survival times provided by the various systemic adjuvant therapies. They claimed it could be due to a type II error, or it may be that there was no difference in survival times between the treatment groups. Post hoc power analysis revealed an exceedingly low statistical power (13.5%) to detect the observed difference based on the sample size. To detect the same difference with 80%

power, inclusion of at least 1,530 dogs would have been required, depending on the allocation ratio between the 2 groups.[150] Bergman stated that immunotherapy, like any form of anticancer treatment, seems to work best in a minimal residual disease setting, suggesting its most appropriate use will be in an adjuvant setting with local tumor therapies, such as surgery and/or radiation.[60]

TABLE 1. Cancer vaccines for melanoma in canine clinical trials

Patients	Control group	Vaccine type	Outcome	Adverse effects	Ref
10	No	Cancer cell vaccine	4/10 response	Minimal or absent	[151]
3	No	DC vaccine	1/3 response	Minimal or absent	[152]
34	No	Cancer cell vaccine	12/34 response	Minimal or absent; vitiligo was observed in 1 dog	[153]
31	Concurrent	Cancer cell vaccine	OS: 370 vs 76 days	Minimal or absent	[154]
58	Published data	DNA vaccine	MST: 464 vs 156 days	Minimal or absent	[155]
58	Published data	DNA vaccine	OS: 476 vs 365 days	Minimal or absent	[156]
24	Retrospective data	DNA vaccine	No difference in survival time	None	[150]
14	Concurrent	DNA vaccine	Survival % at 12m 64.3 vs 15.3	Minimal or absent	[30]
301	Concurrent	DNA and cancer cell vaccine	OS 7x higher for complete tumor removal, 4x higher for partial removal	Minimal or absent	[147]

Minimal adverse effects: *mild induration, erythema, pruritis at injection site;* ***MST:*** *median survival time;* ***OS:*** *overall survival;* ***response:*** *complete response, partial response and/or stable disease (several response options valid as response within same study).*

TABLE 2. Cancer vaccines for lymphoma in canine clinical trials

Patients	Control group	Vaccine type	Outcome	Adverse effects	Ref
11	Concurrent, placebo-controlled	Cancer cell vaccine	OS >348 vs 197.5 days	Abscedations in 50% of patients during 4-6 weeks	[18]
58	Concurrent, randomized	Cancer cell vaccine	No survival benefit	No adverse effects	[157]
26	Concurrent, randomized, placebo-controlled	Cancer cell vaccine	No survival benefit	No adverse effects	[158]
7	No	Cancer cell vaccine	OS suggestive for 2-3x increase in survival	No adverse effects	[159]
14	Recent historical cases from same institution	DNA vaccine (TERT)	OS 403 vs 182 days	No adverse effects elicited by vaccination regimen	[160]
15	Recent published data	Tumor antigen-coated microbeads	No survival benefit	No or mild adverse effects	[161]
19	Recent historical cases from same institution	Tumor RNA-loaded B-cell vaccine	No survival benefit	Acute manageable systemic reaction in one dog. No or mild adverse effects in other dogs.	[162]
21	Concurrent	DNA vaccine (TERT)	OS 205.1 vs 42.7 days	No adverse effects	[149]
19	Concurrent, randomized, placebo-controlled	Cancer cell-based vaccine (autologous tumor lysate with hydroxylapatite + chemotherapy)	Lymphoma-specific survival 505 vs 159 days	No adverse effects	[148]

AI: *auto-immune response;* ***mild adverse effects:*** *mild induration, erythema, pruritis at injection site;* ***OS:*** *overall survival;* ***response:*** *complete response, partial response and/or stable disease (several response options valid as response within same study);* ***TERT:*** *telomerase reverse transcriptase.*

TABLE 3. Cancer vaccines for various other tumor types in canine clinical trials

Tumor type	Patients	Control group	Vaccine type	Outcome	Adverse effects	Ref
Hemangio-sarcoma	28	Recent historical cases	Cancer cell vaccine	OS 182 vs 133 days	No or mild adverse effects	163
Fibrosarcoma	3	No	Cancer cell vaccine	2/3 response	No or mild adverse effects	151
Fibrosarcoma	1	No	DC vaccine	1/1 response	TLS: DIC	164
Fibrosarcoma	8	No	Cancer cell vaccine	OS >1459 vs 184 days	No adverse effects	165
Osteosarcoma	2	No	Cancer cell vaccine	No response	No or mild adverse effects	151
Osteosarcoma	5	No	Cancer cell vaccine	OS >386 vs 96 days	No adverse effects	165
Mammary gland tumor	4	No	DC-vaccine	4/4 response	TLS: cachexia in 1 patient	164
Mammary gland tumor	7	No	DNA vaccine (p62)	7/7 response	No adverse effects	28
Mammary gland tumor	21	No	Cancer cell-based vaccine (Autologous lysate + biotin + streptavidin)	15/21 exceeded expected survival time by 2 weeks to 22 months	No or mild adverse effects	166
Peripheral nerve sheath tumor	9	No	Peptide vaccine (VEGF)	3/9 response	No adverse effects	167
Broncho-alveolar adeno-carcinoma	1	(case report)	Cancer cell vaccine (autologous HSP-rich lysate +Imiquimod + BcG)	1/1 exceeded expected survival time by 40 weeks	No adverse effects	168
Glioma	10	Con-current	DNA vaccine (TetOn Flt3L and herpes simplex virus type I thymidine kinase	OS 341 vs 117 days	No adverse effects	27

			(HSV-tk))			
Glioma	Ongoing	Ongoing	Cancer cell vaccine (autologous tumor lysate)	Ongoing	No severe adverse effects	[27]
Gemistocytic astrocytoma	12	Con-current	Cancer cell vaccine (autologous tumor lysate)	No survival benefit for addition of IFNγ	Temporary left hemiparesis and left blindness after vaccination	[27]
Meningioma	11	Historical	Cancer cell vaccine (autologous tumor lysate)	OS 645 vs 222 days (historical)	No or mild adverse effects	[169]

DIC: *disseminated intravascular clotting;* ***mild adverse effects:*** *mild induration, erythema, pruritis at injection site;* ***OS:*** *overall survival,* ***response:*** *complete response, partial response and/or stable disease (several response options valid as response within same study);* ***TLS:*** *Tumor lysis syndrome.*

3.2 The innate arm of the antitumoral immune response

3.2.1 The general principle of innate antitumoral immune responses

In contrast to the adaptive immune system, the innate immunity is rapidly reacting to aberrant cells or organisms but its activity is typically not very specific. Although the innate immune system is capable of effectively eradicating cancer cells, it can be manipulated by the cancer to contribute to its progression. The innate immunity includes physicochemical barriers (skin and mucosa), blood proteins (such as complement), antigen-presenting and effector cells (DCs, macrophages, neutrophils, γδ T cells (T cells expressing γδ TCRs) and NK cells), and cytokines, which coordinate and regulate the cells involved in innate immunity.[60, 170] Prominent players of the innate immune system will be discussed further in the upcoming sections.

3.2.1.1 Cellular components of the innate immune system

The cellular component of the innate immune system consists of effector cells as well as antigen-presenting cells. The major effector cells of the innate immune system that target cancer cells are NK cells, macrophages, neutrophils and mast cells.[171] NK cells, NKT cells, and γδ T cells can sense malignant transformation and contribute to immune responses at an early stage. All three populations can be activated via NKG2D, through MICA/B and other ligands expressed by cancer cells.[170] The major APCs include DCs and macrophages.[171] Above-mentioned cells are discussed below.

NK cells have the ability to contribute to host control of hematologic cancers as well as solid cancers.[172] NK cells have 3 modes of antitumoral action. (1) They can recognize and kill non-MHC expressing cancer cells[171], [173] and this process depends on 2 functional types of receptors on the NK cell surface: stimulatory receptors and inhibitory receptors. NKG2D, a stimulatory receptor on NK cells, specifically recognizes MICA/B on cancer cells which stimulates cell killing.[172] KIR, an inhibitory receptor on NK cells, detects the presence of MHC class I on cancer cells. Whereas normal cells always carry MHC class I molecules, this is not generally true for cancer cells. When MHC class I is present, KIR will prevent NK cell cytotoxicity.[171] When the MHC class I molecule is not detected, NK cell cytotoxicity is allowed.[171] The final action of a NK cell depends on the activation status of the stimulatory and inhibitory receptor. When the activating signals received by NK cells dominate the inhibitory signals, activated NK cells kill cancer cells directly by releasing perforin and granzymes from cytoplasmic granules, expressing Fas antigen ligand (FasL) or TNF-related, apoptosis-inducing ligand (TRAIL).[174] (2) The second mode of action of NK cells involves interaction with antibodies. When a cancer cell is coated with antibodies against cell-surface antigens, it will bind to the Fc receptor (FcR) present on the NK cell and induce its destruction through ADCC.[171] (3) Finally, the NK cell can contribute to the adaptive arm of the antitumoral immune response by releasing immunostimulatory cytokines and contributing to the release of tumor antigens. In more detail, by secreting IFNγ, activated NK cells can indirectly contribute to CTL activation as well as promote differentiation of Th1 CD4+ T cells.[175] NK cell–mediated cancer cell lysis releases tumor

antigens to DCs and induces DC activation and maturation, for cross-priming of specific T cells.[176, 177]

NK cells can infiltrate the solid tumor microenvironment, and this infiltration has been associated with prolongation of overall survival in cancer patients.[178, 179] Unfortunately, despite the broad array of antitumoral activities of NK cells, cancer cells are able to evade immune surveillance by NK cells through selective survival of NKG2D-ligand-deficient cancer cells. Many cancer cells release soluble NKG2D ligands, which induce anergic or hyporesponsive NK cells as a result of repeated and persistent activation.[170]

NKT cells can be classified into two major functional types: the antitumoral cytotoxic NKT cells and the protumoral immunoregulatory NKT cells. The exerted activity depends on the immunologic context and perhaps on the TCR being used.[172] Cytotoxic NKT cells can be activated directly or indirectly by (recognition of glycolipid antigens presented in CD1d molecules on) cancer cells or IL-12 producing DCs (that express CD1d-associated self-antigens), respectively. Activated cytotoxic NKT cells rapidly produce cytokines (including IL-2, IL-4, and IFNγ) and cytotoxic granules, such as perforins and granzymes that result in antitumoral immune activation and cancer cell death respectively.[170] Similar to NK cells, NKT cells' production of IFNγ indirectly contributes to CTL activation.

In contrast to the antitumoral activity that has been implicated for cytotoxic NKT cells, an immunosuppressive role for immunoregulatory NKT cells has been suggested. The immunosuppressive activity appears to be mediated through production of IL-13 and activation of signal transducer and activator of transcription 6 (STAT6) pathways.[170]

γδ T cells have been implicated in immunomodulation of the tumor micro-environment. Antitumor effects of cytotoxic γδ T cells have been reported, as well as immunosuppressive effects of regulatory γδ T cells.[172] Similar to NK cells and cytotoxic NKT cells, activated cytotoxic γδ T cells can exert cytolytic death of cancer cells through release of cytotoxic granules (containing perforin and granzymes) and effector cytokines (IFNγ and TNFα). Additionally, they express apoptosis-inducing ligands FasL and TRAIL thereby inducing cancer cell death after binding of these ligands to the

cancer cell. Furthermore, as γδ T cells express the CD16-FcR, they can facilitate ADCC. γδ T cells contribute to DC activation, which indirectly supports αβ T cell activation. The antitumoral activity of γδ T cells is suppressed by prostaglandin (Pg)E2 (produced by mesenchymal stem cells), Tregs as well as immunosuppressive factors produced in the tumor microenvironment (TGFβ, PgE2, IDO, soluble NKG2D ligands).[170]

DCs are known as potent professional APCs and form a bridge between the innate and adaptive immune system.[171] The presence of a high number of DCs in the tumor, peritumoral area and/or draining LNs, has been shown to correlate with better disease-specific survival in various solid cancers.[173] DC subsets will not react in a standard predetermined manner, but rather according to the nature of the challenge and/or the form of the antigen or the adjuvant and inflammatory signals.[120, 180] Most importantly, the maturation stage of DCs has an influence on the generated immune response against cancer cells. Immature DCs induce immunosuppression by skewing the patient's T cell response toward a Th2 response type and secreting tolerance-inducing IL-10. On the other hand, mDCs contribute to an antitumoral response by secreting IL-12 and skewing the αβ T cell response toward a Th1 response and prime αβ T cells to the presented antigens.[49] Additionally, mDCs reduce tolerance or expansion of regulatory cells.

Macrophages infiltrating the tumor often participate to local chronic inflammation thus favoring tumor formation and progression.[171, 181] Macrophages are recruited by cancer and stromal cells through the release of various cytokines and chemokines (VEGF, TGFβ, bFGF, M-CSF/CSF-1, uPa, b-defensin-3, Reg3b, oxysterols, CXCL1, CXCL2, CXCL3, CXCL5, CXCL8) whereafter the tumor microenvironment induces a proangiogenic profile in the tumor-associated macrophages (TAM).[181] Under normal circumstances in the tumor environment, the interaction between apoptotic cancer cells and macrophages leads to immune tolerance without provoking significant proinflammatory cytokines.[171] However, tumor-infiltrating macrophages are able to exhibit two contrasting functions, namely antitumoral (TAM1) and protumoral (TAM2) activity. TAM1 produce high amounts of IL-12 and low amounts of IL-10 and

they release chemokines (CXCL9, CXCL10) that attract Th1 cells, whereas TAM2 produce high amounts of IL-10 and low amounts of IL-12 and release chemokines (CXCL17, CCL22) involved in the recruitment of Tregs and Th2 cells.[181, 170] Interestingly, exposure of TAM2 to Th1 cytokines such as IFNγ can induce reeducation into TAM1 cells and abrogate their immunosuppressive abilities.[170, 181]

It is suggested that mast cells may be critical players in tumor progression, promoting angiogenesis and immunosuppression.[182] However, contradictory findings on the role of mast cells in cancer development have been reported. Depending on the immune context, mast cells may have divergent proinflammatory or immunosuppressive effects.[183] Ryan and colleagues reported that many tumor types recruit mast cells and intratumoral mast cells are associated with tumor growth and decreased host survival, possibly through Treg recruitment.[182] In contrast, Heijmans and colleagues observed that dense mast cell infiltration was associated with a favorable outcome in patients with colon cancer.[184] Antitumoral effects of mast cells could be the result of their interaction with antibody-coated cancer cells through the activating and inhibiting FcγR on the mast cell's surface.[171]

As many other immune cells described above, the antitumoral activity of tumor-infiltrated neutrophils depends on its microenvironment.[181] Although neutrophil infiltration is associated with poor clinical outcome, tumor-associated neutrophils (TAN) in early-stage tumors display a more cytotoxic profile toward cancer cells and produce higher levels of TNFα, NO and H_2O_2 than neutrophils in established late-stage tumors. In advanced tumors, these functions are down-regulated and neutrophils acquire a more protumoral phenotype by actively recruiting Tregs and promoting angiogenesis.[181]

Unlike the innate immune cells mentioned above, not much is known about the intratumoral activity of eosinophils and basophils. Eosinophilia is regularly observed during immunotherapy protocols.[185] Additionally, tumor-associated tissue eosinophilia was detected in several types of cancer and linked with a generally good prognostic

value.[185] Eosinophils do often exhibit a state of degranulation when in close proximity to the tumor.[185]

Basophils respond to various IgE-dependent or -independent stimuli, and are engaged in a complex cross-talk with a number of immunocompetent cells.[186] In cancer-bearing patients, the absolute number of circulating basophils is decreased. This appears to be due to the presence of the tumor, since after successful excision of the primary tumor, blood basophil number and blood histamine levels are normal.[187] In general, basophils are able to quickly produce Th2 cytokines (IL-4, IL-13) and express CD40L and chemokine receptors (CCR3).[188] Moreover, basophils are thought to have important regulatory functions in innate as well as adaptive immunity through their contribution to immunoglobulin synthesis and class switching, angiogenesis, autoimmunity and tumor immunity via production of cytokines such as IL-6, VEGF, GM-CSF and IL-3.[186]

3.2.1.2 Non-cellular components of the innate immune system

3.2.1.2.1 Damage-Associated Molecular Patterns (DAMP)

In addition to the direct cancer/innate immune system interactions, a large number of molecules, released as a reaction to cancer cell death, may function as DAMP and interact with innate immune cells (*Table 4*) (*see 3.1.2.1.4*).

TABLE 4. Principal damage-associated molecular patterns (DAMPs)

DAMP on plasma membrane	Effect	Ref
CRT	Stimulates antigen-uptake	87, 141, 189
HSP70	Induces DC maturation (upregulation CD86 and CD40), activates NK cells, attracts monocytes and neutrophils, stimulates antigen uptake	87, 97, 141, 190
HSP90	Induces DC maturation (upregulation CD86 and CD40), activates NK cells, attracts monocytes and neutrophils	87, 190
NKg2D-ligand	Stimulates NK cells, NKT cells and CD8+ T cells to destroy cancer cells that express NKG2D-ligands	97, 141, 191
Secreted		
HMGB1	Induces DC maturation, facilitates processing and presentation of tumor derived antigens, attracts various immune cells	87, 141, 143,, 190
HSP90	Can inhibit activation of TGFβ	87
End-stage degradation products		
ATP	Acts in low concentration as chemoattractant for iDC, induces DC maturation, causes IL-1β release from DC	87, 192
DNA	Stimulates DC and macrophage	141
RNA	Activates innate immune system through TLR3 binding	87

CRT: *Calreticulin;* ***DC:*** *Dendritic cell;* ***HMGB1:*** *High-mobility group box-1;* ***HSP70:*** *Heat shock protein 70;* ***HSP90:*** *Heat shock protein 90;* ***iDC:*** *Immature dendritic cell;* ***NKG2D:*** *Natural killer group 2 member D;* ***TLR3:*** *Toll-like receptor 3.*

3.2.1.2.2 Complement

Complement is a collective term for a system comprising over 50 proteins that function as enzymes, substrates, or regulators of pathway activation. The main functions of the complement are to bridge innate and adaptive immunity, defend the host against bacteria, and bind to immune complexes for complement-mediated cell lysis.[193] Complement can induce cell death by binding antibody complexes, which ultimately leads to cell perforation. There are different mechanisms of complement activation that

differ in their initial steps; yet all lead to the formation of the membrane attack complex (MAC), which ultimately induces chemokines (C3a, C5a) with important inflammatory and chemoattractant functions.[194] Recognition of cancer cells by the complement system is presumed to contribute to immune-mediated elimination of cancer cells.[170]

3.2.1.2.3 Host microbiota

Microbiota can actively contribute to the development of the host's immune response. Indeed, different commensal bacteria can shape a pro- or anti-inflammatory milieu in the intestine. Commensal bacteria can have an antitumoral impact on the systemic immunity.[170] For instance, commensal microbiota will lower the activation threshold for APCs.[170] Furthermore, microbiota can indirectly contribute to an immune-mediated antitumoral response. Cyclophosphamide (CP) causes dysbiosis and translocation of bacteria to LNs, leading to the generation of anti-commensal Th17 cells that, in turn, contribute to the therapeutic efficacy of the drug. When no or few gut bacteria are present, this will impair the generation of Th17 cells and thus the antitumoral effect of CP.[170, 195] Thus, commensals can control various aspects of immunity associated with antitumoral responses.[195] However, the presence of certain microbiota is not always advantageous to the host. An example of tumor progression due to gut microbes includes the development of gastric cancer due to Helicobacter pylori.[170]

3.2.2 Antitumoral application of the innate arm of the immune system via interleukin 12

3.2.2.1 The principles of interleukin 12

IL-12 is an immunostimulatory cytokine, produced by DCs, with a key role in the regulation of inflammation by linking innate and adaptive immune responses.[196] In addition, it has powerful antitumoral properties.[196] The sensing of IL-12 is mediated through the heterodimeric IL-12 receptor (IL-12R) composed of IL-12Rβ1 and IL-12Rβ2.[196] The IL-12R complex is found on NK cells, NKT cells, activated T cells, granulocytes, monocytes and B cells.[197] IL-12Rβ1 is constitutively expressed on naive T

cells, whereas IL-12Rβ2 is only expressed on activated T cells. Co-expression of both receptor subunits is required for the generation of high-affinity binding sites for IL-12. Since naive T cells express IL-12Rβ1 but not IL-12Rβ2 they will not be able to induce antitumoral activity as expression of both receptor subunits is critical for the signal transduction downstream of the receptor complex.[197] As a consequence, IL-12 only stimulates activated T cells,[198, 199] making IL-12 a useful booster of a pre-existing immune response. Subsequent to binding to the IL-12R complex, IL-12 leads to activation and proliferation of T cells and NK cells[200, 201, 202] and is able to recruit NK cells and polymorphonuclear (PMN) cells.[198, 199] In turn, activated T cells produce IFNγ, which induces a positive feedback loop on IL-12 secretion by APCs.[197] Not only can IL-12 engender instant activation of (antitumoral) effector cells, it can also program effector T cells for optimal generation of effector memory T cells and T follicular helper cells.[203, 204] Furthermore, IL-12 inhibits differentiation to and the expansion of Tregs[205, 206] and decreases the suppressive function of myeloid-derived suppressor cells (MDSC).[207] An additional antitumoral mechanism of IL-12 is the inhibition of angiogenesis through mechanisms such as the inhibition of new blood vessel formation by Inducible Protein (IP) 10 and Monokine Induced by Gamma interferon (MIG), cascade products of IFNγ[208] (*Figure 3*). As a result, the production of factors that facilitate new blood vessel formation such as vascular endothelial growth factor (VEGF) and metalloproteinase-9 is decreased.[208, 209, 210, 211, 212]

IL-12 has intriguing antitumoral features, yet grave toxicity has been shown when recombinant IL-12 was administered systemically to human cancer patients.[213] This toxicity has urged the need to find alternatives for systemic IL-12 treatment and led to the evaluation of tumor-specific delivery methods of IL-12. Intratumoral IL-12 production after IL-12 plasmid DNA (pDNA) gene transfer can be accomplished through various ways, one of which is electroporation (EP), a reproducible and highly efficient method to deliver pDNA.[214] Electroporation is the short administration of electrical pulses to ensure the formation of transient pores into the plasma membrane of the cancer cells thereby facilitating intracellular delivery[215] of IL-12 pDNA.[216, 217] By this route, a high intracellular level of IL-12 pDNA can be obtained, leading to production of IL-12 encoded by the plasmid.[216, 217] Electroporation does not cause any

severe adverse events,[218] and several veterinary and human clinical trials showed the safety and clinical efficacy of EP-mediated intratumoral IL-12 pDNA treatments.[219, 220, 221, 222] These results present evidence that it is possible to overcome local suppressor pathways and tip the balance in favor of an antitumor immune response, even with inhibiting effects on non-treated metastases. Regression of primary tumors[222] and metastases[219, 222, 223] has been observed for small tumors after intratumoral IL-12 gene therapy, but successes were present in only a subset of patients.[219, 221, 222, 146]

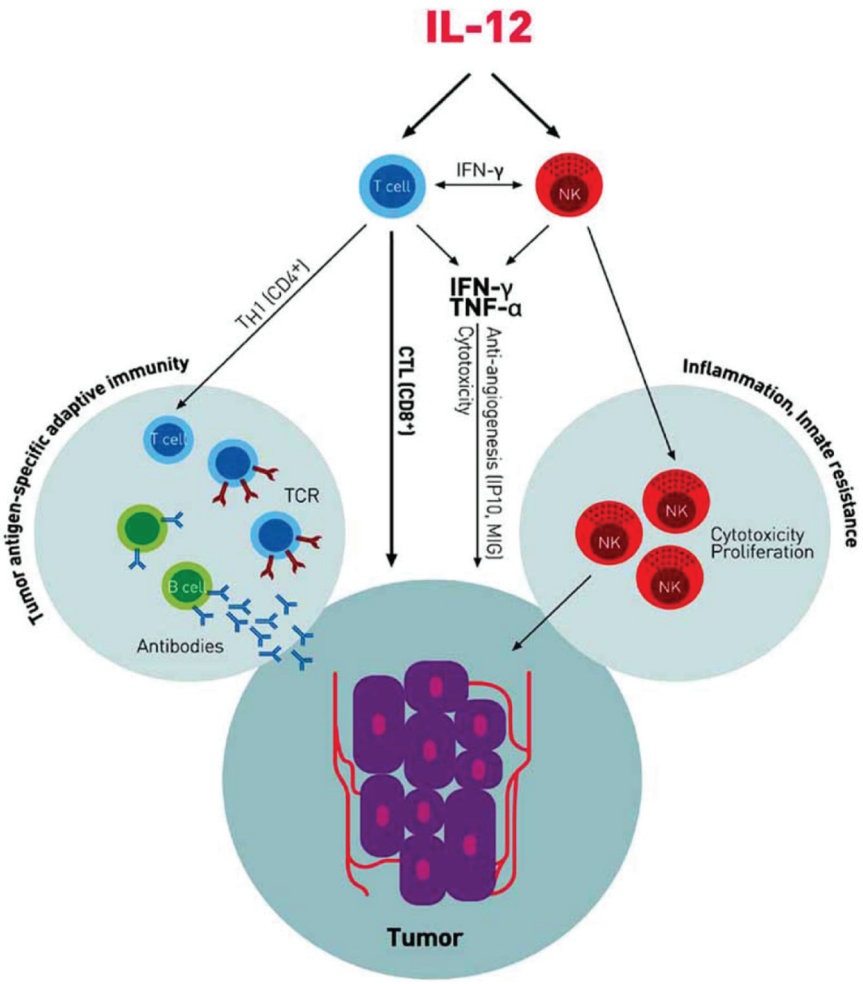

FIGURE 3. Antitumoral mechanisms of IL-12. Adapted from Cemazar 2010 [214]

3.2.2.2 Interleukin 12 in cancer-bearing dogs

3.2.2.2.1 Safety of IL-12 electrogene transfer

So far, 1 intratumoral IL-12 pDNA electrogene transfer (EGT) study was conducted in healthy laboratory dogs with induced tumors[146] and 5 intratumoral IL-12 EGT clinical trials in pet dogs with spontaneous tumors[220, 221, 222, 224, 225, 226] were performed to evaluate the effects of IL-12 EGT and are listed in *Table 5*. No adverse effects were listed for IL-12 EGT in these trials and studies, but fever, anemia, thrombocytopenia, lethargy, poor appetite, cytokine-induced acute vasculitis and subsequent peripheral edema with pleural effusion, and disseminated coagulopathy were reported in dogs treated with a SC injection of an IL-12 immunocytokine (a construct with IL-12 bound to an antibody with specificity for necrotic tissue).[227] These side effects are similar to those reported for early IL-12 clinical trials in human patients, where pure IL-12 was administered IV.[213] IL-12 toxicity is partly explained by the induction of IFNγ through IL-12, resulting in haematologic suppression and gastrointestinal toxicity.[213] Paoloni and colleagues found adverse effects in all dogs with IFNγ serum concentrations of >100 pg/mL.[227] However, the adverse effects may be unrelated to the IFNγ serum concentration as in the IL-12 EGT studies performed so far in dogs, the IFNγ serum concentrations varied between 2.15 -815.6 pg/mL and no serious adverse effects were observed.

No or mild adverse effects for IL-12 EGT were seen in dogs, which is similar to the IL-12 pDNA electroporation trial in humans with metastatic melanoma.[219] The mild adverse events included transient pain during the electroporation procedure and bleeding around the treatment site. In none of the 24 human patients [219] nor 38 canine patients[220, 221, 222, 224, 225, 226] hematologic abnormalities were observed.

In murine melanoma and squamous cell carcinoma tumor models, the toxicity of IL-12 pDNA EGT was evaluated.[228, 229] In the melanoma group, no clinically detectable adverse effects or changes in laboratory parameters were observed. Although no changes in kidney function or biochemical indicators were present, histopathological analysis revealed a focal inflammation and glomerulosclerosis of the kidneys was found 1 month after the procedure.[228] In the squamous cell carcinoma group, a statistically

relevant transient increase of liver enzyme alanine transferase was noticed, as well as a trend in transient decrease in total white blood cell count within 7 days after therapy.[229] In canine patients, only a transient decrease in total white blood cell count was present during 14 days after therapy.[222]

3.2.2.2.2 Clinical responses to intratumoral IL-12 electrogene transfer

Good responses (significant inhibition canine TVT growth followed by complete response) were obtained in the study of Chuang and colleagues in which healthy Beagles were inoculated with canine TVT cells and treated with hIL-12 pDNA when the tumors reached the size of 1-2 cm (after 2-3 weeks).[146] However, such experimental approach does not take into account the effects that slowly growing tumors have on the immune system and, as the authors stated,[146] canine TVT is a more immunogenic tumor than other spontaneous cancers. Indeed, in the later clinical trials in dogs with spontaneous cancer, the responses were less impressive. In Pavlin and colleagues, 8 dogs with mastocytoma's were treated with hIL-12 pDNA electroporation, of which 6 dogs with additional chemotherapy or surgery.[221] The 2 dogs (with mastocytoma) that were treated with solely hIL-12 pDNA demonstrated a stable disease (follow-up time 36 and 44 months respectively), which signifies according to RECIST criteria a response characterized as either an increase or a decrease in tumor burden insufficient in magnitude to qualify as partial regression or progressive disease.[230] Six cancer-bearing dogs with various tumors were treated with hIL-12 pDNA[225] of which 5 dogs with additional antitumoral therapies. The one dog treated with only IL-12 pDNA had an osteosarcoma and IL-12 treatment induced a stable disease (follow-up 5.5 months),[225] whereas median survival for osteosarcoma was 2.6 months after combination of surgery and chemotherapy and 4.3 months after chemotherapy and palliative radiation.[165] Cutrera and colleagues treated 4 cancer-bearing dogs with hIL-12 pDNA as a sole therapy.[222] The response was not measurable in 2 dogs, but partial regression was present in the remaining 2 dogs. In one dog with multiple SCC nodules, 5 nodules were treated intratumorally and a mixed response ensued: 2 partial responses, 1 stable disease and 2 progressive diseases. In the second dog, with an oral melanoma, a transient partial regression was obtained.[222] In a later case series published by the same

research group, 7 cancer-bearing dogs received EGT with only tumor-targeted canine IL-12 pDNA which led to a stable disease (3/7) or partial regression (4/7). Importantly, tumor growth did eventually resume in all patients that were treated with only cIL-12 pDNA, indicating that the antitumor immune response induced by IL-12 is only transient.[224] Recently, 18 dogs with melanoma were treated with subcutaneously injected NHS-IL-12, which led to 2 partial regressions, 5 stable diseases, 8 progressive diseases and 3 euthanasias due to adverse affects.[227]

3.2.2.2.3 Differences in trial design in IL-12 pDNA trials in dogs

The obtained clinical responses are difficult to compare between studies, since different doses or types of IL-12 pDNA, electroporation parameters, treatment frequencies or tumor types were evaluated (*Table 5*). All mentioned parameters can impact the treatment efficiency and will be briefly discussed.

IL-12 of 3 different species has been evaluated in cancer-bearing dogs so far: feline, human and canine IL-12. Human or feline IL-12 pDNA were used in clinical trials due to lack of canine IL-12 pDNA availability. Feline and human IL-12 are both capable of activating canine leukocytes.[220, 231, 232] IL-12 pDNA trials have been conducted with human, feline and canine IL-12 pDNA in dogs with SCC. The outcomes were similar and no serious adverse events were recorded.[220, 222, 224]

Different doses of IL-12 pDNA (between 200 µg and 2 mg) have been evaluated in intratumoral IL-12 pDNA clinical trials in cancer-bearing dogs. For human IL-12 pDNA the best results were observed at 1 mg IL-12 pDNA/tumor (which was the highest dose used in that stud y).[146] Since responses were evident and no adverse effects were recorded with 1 mg of IL-12 pDNA in the preclinical study of Chuang and colleagues, most clinical trials opted to maintain this dose.

The electroporation protocol used to transfect the IL-12 pDNA into the cancer cells can have a significant impact on the transfection efficiency.[233] In a study conducted by Cemazar and colleagues, an electroporation protocol with low voltages and a long duration of pulses resulted in an almost 20 times higher transfection efficiency than a protocol with high voltages and a short duration of pulses.[233] The administration route of IL-12 pDNA significantly impacts the efficacy of the IL-12 treatment. Li and

colleagues claimed that IT but not IM electroporation of the IL-12 gene induces CD8+ T cell infiltration, CTL activity, and tumor eradication.[234] Despite this finding, the effects of IM administration of IL-12 pDNA EGT were still evaluated after this study, as IT IL-12 pDNA administration is not always feasible.[225]

The tumor type has a significant impact on the transfection efficiency as well.[233] In mouse models, melanoma was found to be more easily transfected than carcinoma, followed by carcinosarcoma and sarcoma.[233] The specific properties of the electroporated tumor tissue could elicit different responses to treatment. These properties include differences in tissue organisation, extracellular matrix, presence or absence of necrosis, overall tissue conductivity, the ability of cells to express transfected genes, cell density, and cell size.[233] This could explain why the observed transfection efficiency *in vivo* (1-3%) was significantly lower than *in vitro* (60%).[233] In dogs with cancer, a better clinical effect was found for squamous cell carcinoma (SCC) and acanthomatous ameloblastoma (AA) than sarcoma's,[235] but, due to the small sample size, no statistical significance could be attributed to these findings. The apparently superior effects of IL-12 pDNA in SCC and AA could be pure coincidence, since only 5 SCC's and 2 sarcoma's were treated (*Table 6*).[224]

TABLE 5. Characteristics of IL-12 trials in dogs

Tumor type	IL-12 type	IL-12 admini-stration	IL-12 dose	Electroporation parameters	Treatment frequency	Ref
Mastocytoma	H	IT EP	0.5-1 mg/cm^3/session (tumor volume <2.5 cm^3); 1 mg (tumor volume >2.5 cm^3)	1200 V/cm 100 µs 1x 140 V/cm 8x50ms	Weekly (max 4 sessions)	221
Mastocytoma	H	IM EP	1 mg per tumor	600 V/cm 1x 100 µs, 1 s in between, 80 V/cm 4x 100 ms	Once	225
Mastocytoma*	H	ID EP	1-2 mg per treatment session	1200 V/cm 1x 100 µs, 140 V/cm 1x 400 µs	Once	236

Transmissible Venereal Tumor	H	IT EP	0.1 mg, 0.3 mg or 1 mg per tumor	200 V/cm 10x 50 ms	Once	[146]
Squameus Cell Carcinoma	F	IT EP	150 μg/cm^2 (total amount between 150 and 400 μg)	400V/cm 2x 20 ms	1-3 sessions, 10-day interval	[220]
Squameus Cell Carcinoma	C	IT EP	Not specified	350 V/cm, 2x 20 ms, 100 ms-interval	Variable (depending on results)	[224]
Squameus Cell Carcinoma	H	PT or IT EP	0.2 mg, 0.4 mg, 0.75 mg, 2 mg per treatment session	350 V/cm 2x 20 ms, 100 ms-interval	Day 1 and 7; day 1/7/15; day 1/15/22/29, day 1/8/15/29/35; "multiple"	[222]
Melanoma	H	SC injection, no EP	0.8 mg/m 2 body surface, 1.6 mg/m² and 2.4 mg/m²	NA	Monthly	[227]
Melanoma	H	IT EP	0.2 mg, 0.4 mg, 0.75 mg, 2 mg per treatment session	350 V/cm 2x 20 ms, 100 ms interval	Day 1 and 7; day 1/7/15; day 1/15/22/29, day 1/8/15/29/35; "multiple"	[222]
Melanoma	F	IT EP	150 μg/cm^2 (total amount between 150 and 400 μg)	400V/cm 2x 20 ms	1-3 sessions, 10-day interval	[220]
Sarcoma	C	IT EP	Not specified	350 V/cm, 2x 20 ms, 100 ms interval	Variable (depending on results)	[224]
Sarcoma	F	IT EP	150 μg/cm^2 (total amount between 150 and 400 μg)	400V/cm 2x 20 ms	1-3 sessions, 10-day interval	[220]
Ameloblastoma	F	IT EP	150 μg/cm^2 (total amount between 150 and 400 μg)	400V/cm 2x 20 ms	1-3 sessions, 10-day interval	[220]
Osteosarcoma	H	IM EP	1 mg per tumor	600 V/cm 1x 100	Once	[225]

				µs, 1 s in between, 80 V/cm 4x 100 ms		
Pulmonary histiosarcoma	H	IM EP	1 mg per tumor	600 V/cm 1x 100 µs, 1 s in between, 80 V/cm 4x 100 ms	Once	[225]
Mammary adenocarcinoma (1)	H	IM EP	1 mg per tumor	600 V/cm 1x 100 µs, 1 s in between, 80 V/cm 4x 100 ms	Once	[225]

C: *canine;* ***EP:*** *electroporation;* ***F:*** *feline;* ***H:*** *human,* ***ID:*** *intradermal;* ***IM:*** *intramuscular;* ***IT:*** *intratumoral; ms: millisecond;* ***PT:*** *peritumoral.*

TABLE 6. Outcome in canine IL-12 trials

Tumor type	Patients	Control group	IL-12 type	IL-12 admini-stration	Outcome	Adverse effects	Ref
Mastocytoma°	8 (2)	No	H	IT EP	2/2 response	No adverse effects	[221]
Mastocytoma	6	No	H	IM EP	3/6 response	No adverse effects	[225]
Mastocytoma*	18	No	H	ID EP	16/18 response	No adverse effects	[236]
Transmissible Venereal Tumor	16 (purpose-bred)	Yes	H	IT EP	16/16 response	No adverse effects	[146]
SCC*°	6 (0)	No	F	IT EP	3/6 response	Minimal side effects (loss in appetite, 1 day lethargy)	[220]
SCC	5	Yes	C	IT EP	5/5 transient response	No adverse effects	[224]

SCC	3	No	H	PT or IT EP	1/3 response of primary tumor and anti-metastatic effects (2/3 not evaluable)	No adverse effects	222
Melanoma	18	No	H	Injection SC, no EP	7/18 response, 8/1 progressive disease, 3/18 fatal AE	Thrombo-cytopenia, liver enzymopathies, fever, vasculitis	227
Melanoma	1	No	H	IT EP	1/1 response	No adverse effects	224
Melanoma*°	1(0)	No	F	IT EP	1/1 response	No or minimal adverse effects	220
Sarcoma	2	Yes	C	IT EP	2/2 response	No adverse effects	224
Sarcoma*°	2 (0)	No	F	IT EP	2/2 response	Joint enlargement in 1 dog	220
Ameloblastoma*°	1 (0)	No	F	IT EP	1/1 response	No adverse effects	220
Osteosarcoma	1	No	H	IM EP	1/1 response	No adverse effects	225
Pulmonary histiosarcoma	1	No	H	IM EP	1/1 response	No adverse effects	225
Mammary adenocarcinoma	1	No	H	IM EP	1/1 response	No adverse effects	225

AE: *adverse effects,* ***C:*** *canine,* ***EP:*** *electroporation,* ***F:*** *feline,* ***H:*** *human,* ***IM:*** *intramuscular,* ***IT:*** *intratumoral,* ***PT:*** *peritumoral,* ***response:*** *complete response, partial response and/or stable disease.*

° Dogs between brackets indicate number treated with IL-12 electrogene therapy (EGT) only

** Dogs treated with a mixture of IL-12 pDNA and Bleomycin*

3.3 Cancer-related immunosuppression

3.3.1 The general principles of the immunosuppressive tumor microenvironment

Cancer cells or host cells in the tumor environment can produce immunosuppressive modulators that abrogate the development of an efficient anticancer immune response. Unfortunately for the host, suppressor pathways in cancer immunology generally have a dominant effect over activating stimuli.[45] In this way, a general tolerance toward tumor antigens will be generated when tumor antigens are simultaneously presented by immune-stimulating and by tolerizing APCs.[45] However, exposure of immunosuppressive cells to Th1 cytokines can reeducate immunosuppressive cells and abrogate their immunosuppressive abilities, suggesting that immunosuppression in the whole may be reversible.[172, 181] This is particularly important to cells of the innate immune system, which express stimulatory or regulatory characteristics depending on the immune context. In order to achieve maximal effect of any immunotherapy, the immunosuppressive environment of the tumor has to be tackled.[45, 237]

Two different situations in the tumor microenvironment should be considered. Immunosuppressive mechanisms, such as Treg inhibition or CTLA4 blockage, can address the pre-treatment immunosuppressive burden.[135] Agents such as anti-PDL1-specific monoclonal antibodies, on the other hand, are better suited in the case of post-treatment immunosuppression caused by the stressed patient's cancer cells.[135] Immunotherapy can, via the induction of IFNγ secretion, induce PD-L1 expression on the host's cancer cells and this will suppress the activity of PD1+ T cells.[135]

Not only immunosuppressive cytokines and cancer cells, but also the tumoral stroma actively contributes to a decreased antitumoral immune response. The solid tumor stroma consists of fibroblasts, macrophage-lineage cells and vascular endothelial cells, with variable amounts of extracellular matrix.[238] Cancer cells manipulate the tumor stroma by releasing profibrotic growth factors, such as TGFβ, that inhibit T cell activation, proliferation and differentiation[239] and activate tumor stroma fibroblasts.[240] In turn, the activated fibroblasts induce a shift of the tumor stroma from a defensive to a permissive microenvironment. Indeed, these stromal cells produce growth factors,

such as TGFβ and VEGF, to enhance local tissue growth and further inhibit antitumoral T cell function.[240]

Since multiple factors inhibiting T cell effector functions are present in the tumor microenvironment, blockade of two or more pathways should be considered for optimal therapeutic efficacy of treatments that stimulate the formation of antitumoral effector T cells.[172] Combinations such as blockade of CTLA-4 plus PD-L1 or dual inhibition of LAG-3 and PD-L1 have shown to be synergistic in mouse models.[241, 242] For immunotherapy, the main focus now lies on therapies that enable antitumoral T cell efficacy: ipilimumab is an anti-CTLA-4 antibody that inhibits T cell suppression, pembrolizumab is an anti-PDL1 antibody that inhibits apoptosis of effector T cells by cancer cells and indoximod is small-molecule indoleamine 2,3-dioxygenase (IDO) pathway inhibitor which results in the provision of tryptophan, necessary for effector T cells to function.[243]
Different targeted therapies to challenge the immunosuppressive elements of tumors have been described and they are listed in *Table 7*.

TABLE 7. Immunosuppressive elements in cancer and therapeutic agents that affect their production or function

Target	Means	Ref
Cytokines		
IL-10	Low-dose cyclophosphamide, anti-IL-10/IL-10R-blocking antibodies, anti-IL-10 antisense oligonucleotides	244
Epigenetic modification of DNA and histones		
DNMT HDAC	DNMT-inhibitors (5- azacitidine) HDAC inhibitors (Vorinostat)	64, 245, 246
Growth factors		
EGFR	mAb (Cetuximab, 7A7 mAb)	48, 87, 247
VEGF	mAb (Bevacizumab)	48
TGFβ	mAb, antisense oligonucleotide	137

Homing receptors		
CCR5	CCR5-antagonists (maraviroc, vicriviroc)	[248]
Immune checkpoints		
4-1BB	4-1BB agonistic antibody (BMS-663513)	[48, 134, 249]
CD40	CD40-agonistic antibody (CP-870,893, SGN-40 (Dacetuzumab), HCD 122)	[69, 134, 249, 250]
GITR	DTA-1 agonistic mAb	[249]
ICOS-L	ICOS-L-IgG fusion protein	[134]
OX40	OX40 agonistic antibody	[249]
TRAIL	rhTRAIL, Apomab, Mapatumumab	[53]
Immunosuppressive cells		
Treg	Anti-CTLA4/LAG3 /PD1 (CT-011, BMS-936558)/PDL1 (MDX-1105), CD25 Ab (Ontak), low doses cyclophosphamide, agonistic OX40/GITR (DTA1) Ab, Bcl-2 inhibitor, tyrosine kinase inhibitor (sunitinib)	[70, 251, 249]
MDSC	Formalin-inactivated HSV, chemotherapy, PDL1, stimulation with activated NKT cells	[250, 252, 253]
Immune system down-regulating enzymes		
Arginase	Arginase-inhibitors (shRNA directed against arginase I, S-(2-boronoethyl)-L-cysteine (BEC), NG-hydroxy-L-arginine (NOHA))	[70, 254, 255]
IDO	Methyltryptophan/anti-IDO siRNA, small-molecule IDO pathway inhibitor indoximod	[70, 254, 256]
Melanoma B-raf enzyme	B-raf enzyme inhibitor vemurafenib	[70, 254]
Tyrosine kinase	Tyrosine kinase inhibitors (imatinib/sunitinib)	[70, 254]
Immune system downregulating protein receptors		
A2aR	A2aR mAb, adenosine analogues	[135]
BTLA	BTLA mAb	[135]
CTLA-4	Anti CTLA-4-specific mAb (ipilimumab)	[70, 135, 250, 257]
KIR	KIR-specific mAb	[258]
LAG3	Anti LAG3-specific mAb (IMP701) LAG3–Ig fusion protein (IMP321)	[70, 135, 250, 257]

PD-1	Anti PD1-specific mAb (MDX-1106, CT-011)	70, 134, 135, 250, 257
PD-L1	Anti PDL1-specific mAb (MDX-1105)	70, 135, 250, 257
TIM3	TIM3 mAb	135
Inhibition of apoptosis		
cIAP1	Small molecule antagonists GDC-0152	259
Survivin	Anti-sense oligonucleotide LY2181308, ribozymes, siRNA, small molecule antagonists YM155, chemotargeting with silibinin and NSAID, vaccination with survivin-2B80-88	64, 260
Interaction block		
HVEM and BTLA/CD160	Glycoprotein D Anti-CD160 mAb (CL1-R2)	63, 134
Pathways		
JAK2	ATP competitive inhibitor of JAK2-kinase (AZD1480)	48, 261
mTOR	mTOR inhibitor (aspirin)	251
STAT3	JAK2-inhibitor (AZD1480)	261
Wnt/β-catenin signaling	Wnt/β-catenin signaling inhibitor (Salinomycin)	262
Prostaglandin PgE2		
PgE2	COX2-inhibitor (aspirin, celecoxib, rofecoxib)	137

4-1BB: *Cluster of differentiation 137;* ***A2aR:*** *A2 adenosine receptor;* ***Bcl-2:*** *B cell lymphoma 2;* ***BTLA:*** *B and T lymphocyte attenuator;* ***CCR5:*** *Chemokine receptor 5;* ***cIAP1:*** *Cellular inhibitor of apoptosis 1;* ***CTLA-4:*** *Cytotoxic T lymphocyte-associated antigen 4;* ***DNMT:*** *DNA methyltransferase;* ***EGFR:*** *EGF receptor;* ***GITR:*** *Glucocorticoid-induced TNF receptor;* ***HDAC:*** *Histone deacetylase;* ***HVEM:*** *Herpes virus entry mediator;* ***ICOS L:*** *Inducible co-stimulatory molecule ligand;* ***IDO:*** *Indoleamine 2,3-dioxygenase;* ***Ig:*** *Immunoglobulin;* ***JAK2:*** *Janus kinase 2;* ***KIR:*** *Killer inhibitory receptor;* ***LAG3:*** *Lymphocyte activation gene 3;* ***mAb:*** *Monoclonal antibody;* ***MDSC:*** *Myeloid-derived suppressor cells;* ***OX40:*** *Cluster of differentiation 134;* ***PD1:*** *Programmed death 1;* ***shRNA:*** *Short hairpin RNA;* ***STAT3:*** *Signal transducer and activator of transcription 3;* ***TGF-b:*** *Tumor growth factor b;* ***TIM3:*** *T-cell membrane protein 3;* ***TRAIL:*** *TNF-Related Apoptosis-Inducing Ligand;* ***Wnt/b-catenin:*** *Wingless-type mouse mammary tumor virus integration site family member/b-catenin*

3.3.2 Suppression of the immunosuppressive arm via metronomic chemotherapy

3.3.2.1 The principles of metronomic chemotherapy

Classic chemotherapy is a cytotoxic treatment designed to kill rapidly multiplying cells, such as cancer cells. Unfortunately, due to this rather unspecific approach, this treatment option is accompanied by many side effects.[263] Because of toxicity-issues, the cancer patient requires recuperation time in between treatments, offering the cancer cells the chance to develop escape mechanisms and multiply.[263] An alternative approach for classic chemotherapy is metronomic chemotherapy: administration of chemotherapeutics in small regular doses.[263, 264] Administration of small doses of chemotherapeutics results in very low toxicity allowing uninterrupted administration. In contrast to classic chemotherapy, continuous low doses of certain chemotherapeutics are able to specifically decrease a number of immunosuppressive cells. For instance, low doses of cyclophosphamide and 5-fluoro-uracil can specifically target Tregs and MDSCs respectively.[265, 266] Moreover, both chemotherapeutics have anti-angiogenic effects.[267, 268] This continuous treatment results in persistent suppression of the targeted immune cells and/or tumor vasculature and thus less opportunities for the cancer and endothelial cells to adapt.[267, 269] Whereas rapidly multiplying cancer cells are the target of classic cytotoxic chemotherapy, the immunosuppressive tumor microenvironment is the target of metronomic chemotherapy and thus the intrinsic sensitivity or acquired resistance of the cancer cells to the cytotoxic action of the drug is irrelevant.[263] Additionally, metronomic chemotherapy is very well tolerated and the versatile possibilities of oral intake (and thus at home treatment) contribute to a relatively low treatment-related cost.[270] Although preclinical research and also preliminary clinical results with metronomic chemotherapy are promising, conclusive phase III trials are not yet completed.[263]

Cyclophosphamide is an alkylating prodrug that is activated *in vivo* by liver enzymes to 4-hydroperoxy cyclophosphamide (4-HC).[271] 4-HC induces Treg cell death, due to low intracellular ATP-amount of Tregs, which results in a decreased glutathione synthesis and thus a reduced capacity to detoxify 4-HC.[265] Moreover, 4-HC causes apoptosis of

capillary endothelial cells by up-regulating the endogenous angiogenesis inhibitor thrombospondin (TSP)-1.[272] In turn, TSP-1 binds to the CD36R on endothelial cells inducing apoptosis.[273] Furthermore, TSP-1 binds to the CD47-part of the VEGFR, thus blocking the proangiogenic effects of VEGF on endothelial cells.[274] Metronomic cyclophosphamide has also shown to restore NK cell function and inhibit tumor stromal cells.[275] Chlorambucil and lomustine are both alkylating agents. Similar anti-angiogenic activities as cyclophosphamide have been attributed to low-dose administration of chlorambucil[276] and are so far unknown for lomustine. Toceranib is a tyrosine kinase inhibitor with an antitumoral (targeting tyrosine kinase) as well as anti-angiogenic (targeting VEGFR2 and platelet-derived growth factor receptor (PDGFR)) activity.[277, 278]

3.3.2.2 Metronomic chemotherapy in dogs

3.3.2.2.1 Safety of metronomic chemotherapy in cancer-bearing dogs

Metronomic chemotherapy offers a high tolerability and active modulation of the tumor microenvironment.[279, 280] The most evaluated metronomic chemotherapeutic drug in human and canine patients is cyclophosphamide.[278] Various doses of sustained metronomic cyclophosphamide treatment have been associated with the development of sterile hemorrhagic cystitis in a subset of treated dogs.[278, 281, 282] Cystitis developed as an adverse effect of metronomic cyclophosphamide reported so far was apparent after a minimal treatment duration of 18 weeks.[281] Alternatives for cyclophosphamide evaluated in cancer-bearing dogs include chlorambucil, lomustine and toceranib. It was demonstrated that metronomic toceranib is also capable of specifically targeting Tregs.[280] No cystitis has been attributed to metronomic therapy with chlorambucil, lomustine or toceranib, only mild gastro-intestinal and hematologic effects similar to cyclophosphamide treatment were reported. However, long-term administration (> 12 months) of metronomic chlorambucil or lomustine has led to grave hematologic adverse effects.[283, 284] To summarize, 10 clinical trials evaluating the effects of metronomic chemotherapy have been performed so far. Of those 10 trials, 4 had a control group of which 2 were randomized.[278, 285] The 6 remaining trials did not include

a control group. Seven trials were conducted with metronomic cyclophosphamide, of which 1 was complemented with toceranib[280] and 4 with standard dosing of an NSAID and/or etoposide[281] or toceranib.[278] Treatment was well tolerated, although long-term treatment led to sterile hemorrhagic cystitis in a subgroup of patients. Two trials evaluated metronomic chlorambucil and 1 trial metronomic lomustine. Both metronomic chlorambucil and lomustine were well tolerated with only mild adverse effects. Long-term treatment (over 1 year) did, however, cause grave hematologic adverse effects for both chemotherapeutics (*Table 8*).[283, 284]

3.3.2.2.2 Clinical response of metronomic chemotherapy in cancer-bearing dogs

Significant increases in survival time have been reported after metronomic cyclophosphamide chemotherapy in hemangiosarcoma's or soft tissue sarcoma's,[281, 282, 289] whereas no increase in survival time was found for osteosarcoma's.[278] The effects of metronomic chemotherapy are difficult to evaluate since, in contrast to cytotoxic maximum tolerated dose (MTD) chemotherapy, the objective of metronomic chemotherapy is to offer disease stabilization through modulation of the tumor microenvironment.[286] However, stable disease is a vague parameter for success in immunotherapeutic trials as tumor growth is not constant and has various plateau phases during its progression.[287] These plateau phases are easily confused with stable disease. Therefore, it is difficult to interpret what the true antitumoral value of metronomic chemotherapy is. As a result, other parameters for evaluation of therapeutic success than stable disease might be more appropriate. Since the antitumoral activity of metronomic chemotherapy mainly depends on its anti-angiogenic effects, it has been suggested that angiogenesis markers should be monitored to evaluate treatment efficacy and thus optimal dosing.[286] Various angiogenesis markers have been proposed, including TSP-1, VEGF and circulating endothelial progenitor (CEP) cells.

Metronomic chemotherapy has much potential in combinatorial treatments[263] and possibly synergizes with other immunomodulatory or anti-angiogenic antitumoral therapies.[286] Metronomic chemotherapy may sensitize cancer (stem) cells to classic

cytotoxic chemotherapy.[282] As a consequence, dogs receiving continuous metronomic chemotherapy may benefit from pulses of conventional high-dose chemotherapy.[282] It may not be opportune to continue metronomic chemotherapy during classic cytotoxic chemotherapy, as metronomic chemotherapy during classic cytotoxic chemotherapy did not result in specific decrease of Tregs.[275] However, specific decrease of Tregs represents only one of the effects associated with metronomic chemotherapy and lack thereof during classic cytotoxic chemotherapy does not exclude its other antitumoral effects (such as anti-angiogenesis, restoration of NK cell effector functions and inhibition of normal stromal cells that support the growth of cancer cells).[275]

TABLE 8. Metronomic chemotherapy trials in dogs with spontaneous cancer

Tumor type	Patients	Control group	Treatment protocol	Outcome	Adverse effects	Ref
Stage II splenic hemangio-sarcoma	9	3 randomized + 21 historical doxorubicin treated dogs	Surgery followed by CP 12.5-25mg/m² + etoposide 50mg/m²+ piroxicam 0.3mg/kg	MST 178 days vs 133 days*	2/9 sterile haemorrhagic cystitis, mild unspecified gastro-intestinal or hematologic adverse effects	[281]
Incomplete resected soft tissue sarcoma	30	Concurrent non-randomized dogs	CP 10mg/m²+piroxicam 0.3mg/kg	Median DFI 410 vs 211 days*	3/30 sterile haemorrhagic cystitis, mild gastro-intestinal or renal adverse effects	[282]
Soft tissue sarcoma	11	No	CP 12.5mg/m² or CP 15mg/m²	MVD and Treg decreased (15mg/m²)	No adverse effects	[279]
Various metastasized cancers	15	No	CP 25mg/m² + celexocib 2mg/kg	6/15 response (MST 102 days)	No adverse effects	[288]

Various advanced stage cancers	15	No	Toceranib 2.75mg/m² + CP 15mg/m²	Treg decreased, serum IFNγ increased	Mild gastro-intestinal or bone marrow-related adverse effects	[280]
Macroscopic soft tissue sarcoma	20	Historic non-randomized dogs	Surgery followed by radiotherapy followed by 1-2 mg/kg/day thalidomide + piroxicam 0.3 mg/kg/day + 7 mg/m² CP/ 2 days	OS 757 vs 286 days*	No or mild adverse effects	[289]
Various cancers	30	Concurrent randomized	CP 12.5 mg/m², TMZ 6.6 mg/m², CP 12.5+ TMZ 6.6 mg/m²	Treg decrease for CP 12.5 mg/m² and its combination	2/30 sterile haemorrhagic cystitis	[285]
Osteosarcoma	126	Concurrent randomized surgery followed by carboplatin followed by CP+PI, historic surgery and carboplatin only	Surgery followed by carboplatin treatment followed by CP 10 mg/m² + 0.3 mg/kg piroxicam + 2.75 mg/kg toceranib	OS no significant difference	7/126 sterile haemorrhagic cystitis, mild gastro-intestinal, hematologic adverse effects	[278]
Various cancers	81	No	Lomustine 2.84mg/m²	29/81 response	22/81 discontinued treatment due to mild hematologic, gastrointestinal, and/or moderate biochemical negative effects	[283]

Various cancers	36	No	Chlorambucil 4mg/m²	18/36 response	Mild gastrointestinal toxicity in 4 dogs	[290]
Transitional cell carcinoma	31	No	Chlorambucil 4mg/m²	21/31 response (MST 221 days)	Mild gastro-intestinal adverse effects, mild lethargy. 1/20 mild anemia, mild neutropenia, severe thrombocytopenia after 20 months	[284]

CP: *cyclophosphamide,* ***DFI:*** *disease-free interval,* ***MST:*** *median survival time,* ***MTTE:*** *median time to terminal event,* ***MVD:*** *mean vascular density,* ***TMZ:*** *temozolomide.* ***Mild adverse effects:*** *anorexia, nausea or diarrhoea*

*** indicates a significant difference*

3.4 Combinatorial immunotherapy against cancer

Two major tumor microenvironments exist: T cell infiltrated and non-T cell infiltrated.[170, 172] The T cell infiltrated tumor indicates that the tumor did induce an immune response, allowing antitumoral T cells to infiltrate the tumor. Unfortunately, the antitumoral effect of the infiltrated T cells is neutralized through inhibitory mechanisms of the tumor (such as secretion of immunosuppressive cytokines by cancer cells or immunosuppressive immune cells). On the other hand, tumors that are not infiltrated by T cells never evoked an antitumoral immune response.[170, 172] One could adapt an antitumoral treatment strategy according to the patient's tumor T cell infiltration status. For patients with a T cell infiltrated microenvironment, it could be sufficient to merely reactivate the immune response, whereas the immune response in patients with the non-T cell infiltrated microenvironment remains to be generated.[170] The differences in tumor microenvironment may explain why in most immunotherapeutic trials only a subset of patients responds to the treatment.[165, 219, 220] Indeed, most trials evaluate the effects of one immunotherapeutic treatment regimen wherein only one arm of the antitumoral immune system is stimulated, which is likely

insufficient for most cancer patients. It is unrealistic to expect significant results from one type of immunotherapy as the immune system and the tumor microenvironment interact in a very complex way. Most successful immune therapies are (just as in MTD chemotherapy[291]) combinatorial in nature. To treat against both T cell infiltrated and non-T cell infiltrated tumor microenvironments, a combinatorial approach targeting immune activation, immune enhancement and inhibitors of effector T cells might be most appropriate.[170, 292] Since immunotherapy is only effective if the cancer microenvironment is restructured,[59] depletion of Tregs and/or MDSCs from the tumor microenvironment could allow cancer vaccines not only to induce proper systemic effector and memory T cell populations, but also allow these effector cells to migrate to the cancer sites and exert their antitumoral activity.[59]

The most effective cancer therapies probably consist of combinations of various immunotherapeutic strategies with rational combinations of other standard therapies (such as surgery, radiotherapy and conventional chemotherapy).[292] For cancer vaccines in dogs, the best results so far were obtained by combining surgery with a cancer vaccine and antitumoral cytokines.[147]

4 Conclusion

The interaction between the immune system and the tumor microenvironment is very complex. In cancer patients, many aspects of the immune system contribute to the final therapeutic outcome which may be to the advantage or disadvantage of the cancer patient. Since the immune context is very important for the behavior of tumor-infiltrating immune cells, efforts should be directed toward the manipulation of the protumoral microenvironment into an antitumoral environment capable of activating immune cells toward the tumor. It is therefore interesting to improve our knowledge on the most important aspects of the immune system in immunotherapeutic treatments: the innate immune system, the adaptive immune system and the immunosuppressive regulators of the immune system.

SCIENTIFIC AIMS

The general aim of this thesis was to explore the efficacy and tolerability of an antitumoral combination immunotherapy in cancer-bearing pet dogs.

In the Laboratory of Gene Therapy, a preclinical combinatorial immunotherapeutic study was successfully performed in mice by Denies and colleagues.[293] The combination of intratumoral murine (m)IL-12 pDNA administration with metronomic cyclophosphamide and a tyrosinase vaccine resulted in a higher cure rate and survival of cancer-bearing mice than only intratumoral mIL-12 pDNA administration or the combination of intratumoral mIL-12 pDNA with metronomic cyclophosphamide. We wanted to evaluate the safety and efficacy of each component in a more appropriate cancer model for humans, namely cancer-bearing pet dogs, prior to combination therapy.

Chapter 1. Since the tyrosinase vaccine would only be applicable to dogs with melanoma, a vaccine vehicle for all tumor types (cancer cell vaccines based on immunogenically killed cancer cells) was first researched in mice. Immunogenically killed cancer cells are cancer cells that are killed with immunogenic cell death-inducers, which cause the cancer cells to evoke an immune response upon their death as opposed to a tolerance response.
Afterward, the aim was to evaluate the effects of each component of the aforementioned combination treatment in cancer-bearing pet dogs.[293]
Chapter 2. The safety and efficacy of only intratumoral IL-12 pDNA administration were evaluated in cancer-bearing pet dogs.
Chapter 3. The effects of administration of only metronomic cyclophosphamide in cancer-bearing pet dogs were already assessed by Denies and colleagues[293] prior to the studies described in this thesis. The results from this study indicated that the used metronomic cyclophosphamide protocol was safe and effective at reducing the amount of circulating Tregs. Therefore, this metronomic cyclophosphamide protocol was selected for our combination treatment of intratumoral IL-12 pDNA administration with metronomic cyclophosphamide in cancer-bearing pet dogs and the safety and efficacy of this combination were assessed.

RESEARCH STUDIES

CHAPTER 1

Can dendritic cells improve whole cancer cell vaccines based on immunogenically killed cancer cells?

This chapter is based on

Cicchelero L, Denies S, Devriendt B, de Rooster H, Sanders NN. Can dendritic cells improve whole cancer cell vaccines based on immunogenically killed cancer cells? OncoImmunology 2015; 4 (12): e1048413-1-9.

1 Abstract

Immunogenic cell death offers interesting opportunities in cancer cell vaccine manufacture, as it increases the immunogenicity of the dead cancer cell. Furthermore, fusion of cancer cells with dendritic cells is considered a superior method for generating whole cancer cell vaccines. Therefore, in this work, we determined in naive mice whether immunogenically killed cancer cells per se (cancer cell vaccine) elicit an antitumoral immune response different from the response observed when immunogenically killed cancer cells are associated with dendritic cells through fusion (fusion vaccine) or through co-incubation (co-incubation vaccine). After tumor inoculation, the type of immune response in the prophylactically vaccinated mice differed between the groups. In more detail, fusion vaccines elicited a humoral anticancer response, whereas the co-incubation and cancer cell vaccine mainly induced a cellular response. Despite these differences, all three approaches offered prophylactic protection against tumor development in the murine mammary carcinoma model tested.

In summary, it can be concluded that whole cancer cell vaccines based on immunogenically killed cancer cells may not necessarily require association with dendritic cells to elicit a protective anticancer immune response. If this finding can be endorsed in other cancer models, the manufacture of cancer cell vaccines would greatly benefit from this new insight, as production of dendritic cell-based vaccines is laborious, time-consuming and expensive.

2 Introduction

Cancer vaccines have emerged as a new therapeutic modality for cancer.[3] Cancer vaccines aim to reeducate the immune system to recognize and eliminate cancer cells.[63] An additional aim of cancer vaccines is to create an immune memory in order to prevent cancer recurrence. As a single modality, cancer vaccines will in most cases not be able to eradicate established tumors.[145] However, they can reduce the risk of cancer recurrence,[294] increase survival time[154, 295] and induce regression of metastases.[151, 296]

One can divide cancer vaccines into (whole) cancer cell vaccines, genetic vaccines and peptide or protein vaccines.[297] There are some strong arguments to consider whole cancer cell vaccines as superior to most other cancer vaccine types.[297] First of all, there is no need to identify tumor antigens or select immunodominant epitopes on the tumor antigens for vaccine generation. Furthermore, a great variety of antigens is offered by whole cancer cells which evoke a Major Histocompatibility Complex (MHC)-independent broad-ranged anticancer response. Additionally, the cancer cells contain epitopes for parallel presentation to both CD8+ and CD4+ T cells, which greatly diminishes the risk of tumor escape. Autologous cancer cells have the added advantage of containing patient-specific unique mutated antigens. Whole cancer cell vaccines have shown a higher objective clinical response ratio than peptide, protein or genetic vaccines.[66, 69, 70, 71] Despite observed tumor eradication in experimental animal studies, limited success has been obtained so far in clinical trials for whole cancer cell vaccines in humans.[71, 100]

Various forms of cancer cell vaccines exist and those loaded *ex vivo* with dendritic cells (DC) (DC-based vaccines) have been considered superior to non-DC-based vaccines in stimulating anticancer immunity *in vivo*[101] as antigen presentation is facilitated in the former.[67] DC-based vaccines can be generated through various techniques. DCs can be loaded through co-incubation with different types of tumor antigens, namely, whole cancer cells, tumor lysate, apoptotic cancer cells, peptides, transfection of RNA or DNA[68] or, alternatively, they can be fused with cancer cells.[298] DCs fused to cancer cells elicit levels of DC maturation and tumor-specific T cell activation that are superior to the levels obtained by DCs co-incubated or pulsed with cancer cells or tumor lysate, respectively.[296, 298] In spite of these advantages, fusion vaccines are not common practice, since fusion is the most complicated method of loading DCs with cancer cells and has a low production efficiency.[296]

The immunogenicity of whole cancer cells in vaccines can be increased through immunogenic cell death (ICD).[299] In analogy, vaccines based on DCs pulsed with apoptotic cells are considered superior to those pulsed with whole cell lysate or necrotic

cells.[298, 300] ICD can be induced by chemical agents such as anthracyclines (eg. Mitoxantrone (MTX)) or by physical stimuli (eg. UV light or γ-irradiation). The main characteristic of ICD-inducing stimuli is their ability to induce the expression or release of Danger (or damage)-Associated Molecular Patterns (DAMPs) from the killed cells. The DAMPs that are expressed or released by the cancer cells after ICD can interact with Pattern Recognition Receptors (PRRs) present on immune cells. Binding of DAMPs to PRRs results in the release of immunostimulatory cytokines and chemokines by the immune cell. As a result, antigen presenting cells (APCs) are stimulated to efficiently take up and process tumor antigens and cross-prime T cells.[299]

In this article, we examined the *in vivo* effects of vaccines based on immunogenically killed cancer cells, induced by MTX, and whether the protective anticancer effects could be augmented through association with DCs (through co-incubation or fusion of DCs with immunogenically killed cancer cells).

3 Materials and Methods

3.1 Animals

Female C57BL/6 mice, aged 6-8 weeks, were purchased from Janvier Labs (Janvier Breeding Center, 2013-06-ENG-RM-20) and housed at the Laboratory for Gene Therapy at the University of Ghent. Experiments were conducted at age 16-52 weeks. This experiment was approved by the Ethical Committee (approval number EC2013/123).

3.2 Media and cell culture

Complete medium consisted of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, 21041-033), supplemented with 100 IU/ml penicillin (Invitrogen, 15140-122) and 10% heat-inactivated fetal bovine serum (Thermoscientific, SV30160.03).

EO771 cells, an aggressive estrogen receptor-positive poorly immunogenic mamma adenocarcinoma cell line (a kind gift from professor Jo Van Ginderachter), were grown in culture medium (CM). Stable transduction with luciferase of these cells with a luciferase gene was performed as described earlier by Van Impe.[301] Briefly, retrovirus was produced in HEK293T cells by calcium phosphate transfection, harvested after 48 and 72 h, filtered and concentrated by ultracentrifugation. Retroviral transduction was performed in the presence of 8 μg/ml polybrene and the efficiency was evaluated by bioluminescence. These cells were grown in DMEM, supplemented with 10% fetal bovine serum, 100 mg/ml streptomycin, 100 IU/ml penicillin and 1 mmol/ml L-glutamine (Invitrogen, 25030-081).

Monocyte-derived DCs were generated as described by Lutz.[302] Briefly, bone marrow cells were harvested from the femur and tibia of sacrificed mice and seeded at $2x10^6$ cells per 100 mm dish in 10 ml DC-CM (ie. CM with 20 ng/ml recombinant murine GM-CSF (Peprotech, 315-03) and 50 μM beta-mercaptoethanol (Sigma-Aldrich, M3148-25ML)). Five ml of DC-CM was added on day 3 and on days 6 and 8 collected, centrifuged after which the cell pellet was resuspended in 5 ml of fresh DC-CM and given back to the original plate. On day 10, non-adherent DCs were harvested by gentle pipetting.

3.3 *In vitro* evaluation of immunogenic cell death

Induction of ICD. EO771 cells were incubated for 2 h in SFM (DMEM, supplemented with 100 IU/ml penicillin) containing various concentrations of MTX (Sigma, M6545) (1, 10 or 20 μM). Afterwards the MTX-treated EO771 cells were immediately analyzed for expression of the ICD markers CRT (Millipore, MABT145) and HSP70 (Abcam, ab45133) through Anti-Rabbit Antibody (Invitrogen, A-11008), or further incubated during 22 h in the same MTX-containing medium or in SFM. As controls, EO771 cells were incubated either in SFM or in CM during 2 or 24 h (n= 4 for each condition tested). Expression of ICD markers on cells was analyzed by flow cytometry (Accuri C6, BD Biosciences). To generate the cell suspension for flow cytometry we compared forceful pipetting with trypsinization, and we found that cells harvested via

trypsinization had a significantly lower expression of surface associated DAMPs than cells harvested via forceful pipetting (data not shown). Hence, all further cell harvesting was done through forceful pipetting.

Evaluation of cell death after Mitoxantrone treatment. Vaccines based on cancer cells should be free of multiplying cancer cells to eliminate the risk of inducing cancer after *in vivo* administration. For this purpose, cell death after MTX-treatment was evaluated *in vitro*. Luciferase-positive EO771 cells ($1x10^4$) were treated with 1 µM MTX in 100 µl CM or left untreated in 100 µl CM in a black 96-well plate. After 24 h, 48 h and 72 h of incubation 10 µl of D-luciferin (Gold Biotechnology, LUCK-1G) was added per well and the plate was incubated at 37°C and 5% CO2 and 95% relative humidity for 15 min. The expression of luciferase (as identification of living EO771 cells) was measured with an IVIS Lumina II (Perkin-Elmer, Zaventem).

Preparation of vaccines. Cancer cell-DC fusion hybrids were prepared as follows. Bone marrow-derived DCs and MTX-treated EO771 cells were mixed before fusion at DC:CC ratio (2:1) using 50% polyethylene glycol (PEG) (Sigma, P7306), as previously described.[303] Briefly, the DCs and MTX-treated EO771 cells were washed with DMEM and mixed before fusion at DC:CC ratio (2:1). The cell mix was spun for 5 min at 1300 rpm after which the supernatant was removed. The pellet was broken by a 2 min pipetting in 1 ml of prewarmed PEG (37˚C). Then, 1 ml of prewarmed DMEM was slowly added over 1 min followed by slow addition of 3 ml of DMEM over a period of 3 min. This was followed by slow addition of 10 ml DMEM over a period of 1 min. The mixture was afterwards incubated for 5 min at 37˚C in a 15 ml final volume of DMEM, followed by centrifugation for 5 min at 1500 rpm. The cell pellet of the fusion products was resuspended in 30 ml CM, unless otherwise specified, and plated in 3 100 mm Petri dishes for 1 h. After 1 h of incubation, (fused) DCs were magnetically separated from the MTX-treated EO771 cells in the CC-DC mix according to the manufacturer's instructions. Briefly, DCs were targeted with an antibody complex recognizing CD11c and dextran-coated magnetic particles (Stemcell Technologies, 18780). Labeled cells were subsequently separated using an EasySep™ magnet (Stemcell Technologies, 18000).

Cancer cell-DC hybrids were also generated via co-incubation of MTX-treated EO771 cells with DCs (co-incubation vaccine). In more detail, non-PEG-treated DCs and EO771 cells were co-cultured at the same DC:TC ratio (2:1). Unless otherwise stated, co-incubated cells were harvested 24 h after co-incubation. Like the fusion CC-DC hybrids the co-incubated CC-DC hybrids were magnetically separated from the CC-DC mix prior to further use.

Confirmation of hybrid formation. Before fusion or co-incubation the DCs and MTX-treated EO771 cells were fluorescently labeled using respectively the PKH67-Green Fluorescent Cell Linker Kit (Sigma, mini67 1 kit) and the CellVue® Claret Far Red Fluorescent Cell linker Kit (Sigma, MINCLARET-1KT). After labeling, the DCs and EO771 cells were mixed (DC:CC ratio 2:1) and were fused with 50% PEG or co-incubated as described above.

The fusion and phagocytosis efficiency (during co-incubation) was evaluated by assessing the percent of double-stained cells using flow cytometry and an inverted fluorescence microscopy (Ti-S/L100). Prior to vaccine administration, the amount of hybrids was determined through flow cytometry. A gate was placed on CD11c-PE-FL2 positive cells and within this gate the number of MTX-FL4 positive cells was determined.

Influence of fusion or co-incubation on DC maturation. After staining with CD11c antibody (Biolegend, 117307), the phenotypical maturation markers CD40 (eBioscience, 11-0402) and CD86 (eBioscience, 11-0862-81) and the functional maturation marker IL-12p70 (eBioscience, 53-7123-80) were used to stain naive DCs, DCs co-incubated with LPS (Sigma, L4391-1MG) (100 ng/ml for 22 h), DCs co-incubated with MTX-treated EO771 cells and DCs fused to MTX-treated EO771 cells. In the latter 2 conditions the amount of maturation markers was determined after magnetic separation of the CC-DC hybrids. For IL-12 measurement, the cells were permeabilized. Briefly, the cells were incubated during 4 h with Brefeldin A (Biolegend, 420601) to block secretion of cytokines and then stained with CD11c prior to fixation. Afterwards the cells were permeabilized and stained with anti-IL-12 antibody in permeabilization buffer for 30 min at 4°C, and then analyzed by flow cytometry.

***In vivo* experimental setup.** In the vaccine groups, mice were SC vaccinated on day 0 in the left flank with $2x10^6$ MTX-treated EO771 cells (cancer cell vaccine group), with $2x10^6$ MTX-treated EO771-DC hybrids obtained through co-incubation (co-incubation vaccine group), or with $2x10^6$ MTX-treated EO771-DC hybrids obtained through fusion (fusion vaccine group). On day 35, a second vaccine was given in the right flank. The control group consisted of mice injected with a phosphate buffered saline solution (DPBS) (Invitrogen, 14190250) on days 0 and 35 (SC) in the flanks (left and right flank, respectively). The injections were performed with a hypodermic needle. Three mice in each group were euthanized on day 40 to characterize the cellular as well as the humoral response. Tumor inoculation was performed on day 42 with $5x10^5$ EO771 cells in the right mammary fat pad of the fourth mammary gland with a hypodermic needle. Eight mice in each group were euthanized on day 142 (or earlier if the tumor would reach 1 cm^2) for the memory cellular and humoral response. After euthanasia, spleen and serum were harvested (*Figure 8*).

Cellular response: tumor-specific cytotoxicity. Splenocytes ($2x10^5$) from every mouse from every treatment group were co-incubated with luciferase-positive EO771 cells ($1x10^4$) in 100 µl CM in a black 96-well plate. After 24 h of co-incubation, 10 µl of D-luciferin (Gold Biotechnology, LUCK-1G) was added per well and incubated during 15 min at 37°C, 5% CO_2 and 95% relative humidity. The expression of luciferase was measured with an IVIS Lumina II (Perkin-Elmer) and compared between the different treatment groups. The expression of luciferase is inversely correlated to cancer cell death caused by tumor-specific cytotoxicity.

Cellular response: activation of Th1 or Th2 effector T cells. The ability of splenocytes of vaccinated mice to produce IFNγ (marker for activated CTLs and Th1 cells) or IL-4 (marker for activated Th2 cells) when stimulated with EO771 whole cell lysate was evaluated. In detail, whole cell lysate of EO771 cells was developed as follows: cells were harvested with a scraper and re-suspended in a DPBS solution, at a final concentration of $8x10^7$ cells/ml. A tablet cOmplete Mini (Roche, 5892970001) was added to the cells and the mixture was frozen quickly in a -154°C freezer for 10 min followed by rapid thawing in a 37°C water bath for 5 min. The quick freeze/thaw process was performed 5 times.

Subsequently, splenocytes were harvested and $1x10^7$ splenocytes per mouse were incubated in 100 µl CM with 50 µl tumor lysate (the equivalent of $4x10^6$ tumor cells) or without tumor lysate. After 18 h of incubation, Brefeldin A (1/1000 dilution) was added to block secretion of cytokines and the splenocytes were further incubated during 4 h. Afterwards, the splenocytes were harvested, washed and resuspended in FACS Buffer (BD Biosciences, 554656). Cell-surface staining with CD8 antibody (Biolegend, 100705) or CD4 antibody (Biolegend, 100509) was followed by fixation, permabilization and intracellular staining with IL-4 antibody (Biolegend, 504103) and IFNγ antibody (Biolegend, 17-7311-81). The cells were analyzed with a flow cytometer and events in the lymphocyte gate were selected. Control for specificity of the cytokine response was evaluated by assessing non-stimulated splenocytes.

Humoral response. The presence of tumor-specific antibodies was determined as previously described [293]. Briefly, serum of each mouse was 1/8 diluted in FACS Buffer. Washed EO771 cells ($1.25x10^5$) were resuspended in 50 µl of this diluted serum and incubated for 1 h at 37°C, 5% CO2 and 95% relative humidity. Subsequently, the cells were washed and stained with anti-mouse IgG antibodies (Biolegend, 405308) and the number of positive cells was analyzed via flow cytometry. Control for specificity of staining included EO771 cells that were not incubated with serum before being stained with the secondary antibodies.

3.4 Statistical Analysis

All data were analyzed with the statistical software program SPSS (version 19.0). The parametric data were analyzed with one-way ANOVA, the non-parametric data with the Kruskall-Wallis test with Bonferroni correction. Proportion data were analyzed with Fisher's exact test. Statistical significance was determined at $p \leq 0.05$.

4 Results

***In vitro* optimization.** Different MTX concentrations and incubation conditions were tested to induce ICD of the EO771 cells. The most optimal protocol for this purpose

was 2 h of incubation in a 1 μM MTX-containing serum-free medium (SFM) followed by 22 h of incubation in SFM. This protocol yielded the highest expression of Calreticulin (CRT) (35.39% ±16.7) and Heat Shock Protein 70 (HSP70) (50.64% ±20.74) on the surface of the immunogenically killed cancer cells. These MTX-treated cells also expressed significantly more CRT and HSP70 than the mildly stressed cells that were incubated in SFM (14.87% ±9.63, p=0.01 and 23.44% ±12.12, p= 0.012 respectively) and than the unstressed control cells that were incubated in culture medium (CM) (12.1% ±3.2, p= 0.003 and 17.49% ±12.02, p=0.003 respectively).
Mitoxantrone-treated EO771 cells are not able to induce tumors, since it was confirmed *in vitro* that EO771 cells treated with MTX do no longer multiply and die over a period of 3-4 days, whereas untreated EO771 cells continue to multiply.
We demonstrated that immunogenic cell death has a positive influence on phagocytosis. We followed the phagocytosis of untreated and MTX-treated EO771 cells by DCs during 12 h. MTX-treated EO771 cells were much faster phagocytized by DCs than untreated EO771 cells. Depending on the time point, 2- 2.5 times more cancer cells (CC)-DC hybrids were formed after co-incubation of DCs with MTX-treated EO771 cells than with untreated EO771 cells (*Figure 4*).

Subsequently, CC-DC hybrid formation via fusion or co-incubation of immunogenically killed cancer cells with DCs was compared and followed over 72 h. Thirty min after fusion or co-incubation, a significantly higher percentage of double-fluorescent (hybrids) cells was observed after fusion (p= 0.002). The instant formation of hybrids after fusion was confirmed by the observation that the generation of hybrids after co-incubation occurred much slower. Indeed, the percentage of hybrids in the co-incubation group gradually increased as a function of time and after 24 h, the percentage of hybrids was the same as in the fusion group (*Figure 5*).

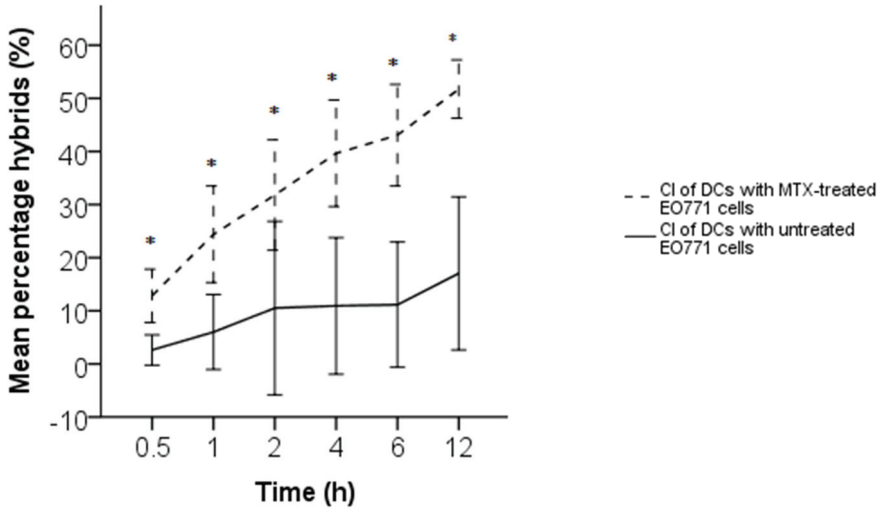

FIGURE 4. Phagocytosis of MTX-treated and untreated EO771 cells. Phagocytosis of MTX-treated (dash line) EO771 cells and untreated (solid line) EO771 cells by DCs during 12 h of co-incubation (n= 4, error bars ± 1 SD)

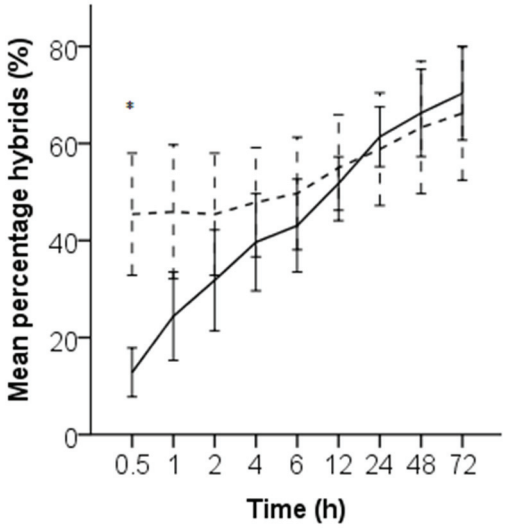

FIGURE 5. Formation and characterization of CC-DC hybrids. Hybrid formation between MTX-treated EO771 cells fused to DCs (dash line) or co-incubated with DCs (solid line) (n= 4, error bars ±1 SD).

To ensure that real hybrids were measured and not merely aggregated cells, the formation of hybrids was also confirmed through fluorescence microscope imaging (data not shown).

One can expect DCs to mature after fusion or co-incubation with immunogenically killed cancer cells.[87,97,141,190] However, maturation markers such as CD40 and CD86 can also be expressed by cancer cells.[304] Therefore, to unambiguously confirm DC maturation, the expression of CD40, CD86 and IL-12 by MTX-treated and untreated EO771 cells was measured. CD40 was highly expressed by MTX-treated and untreated EO771 cells, while CD86 and IL-12 were barely expressed by treated as well as untreated EO771 cells. Interestingly, MTX-treatment seemed to increase the expression of CD40, although statistical significance could not be reached ($p=0.053$), while it did not affect the expression of CD86 and IL-12 ($p= 0.318$ and $p= 0.912$, respectively).

Since it was observed that the maturation marker CD40 was highly expressed on MTX-treated cancer cells, this marker was not optimal to evaluate maturation of CC-DC hybrids. Hence CD86 and IL-12 were used as maturation markers (*Figure 6* and *Figure 7*). Twenty-four h after fusion or co-incubation with immunogenically killed EO771 cells, CD86 expression as well as IL-12 production in DCs were not significantly higher than in naive DCs and did not reach the CD86 and IL-12 levels observed in LPS treated DCs. After 48 h, the CD86 expression remained not significantly higher and, interestingly, the level of IL-12 expression dropped in DCs fused with immunogenically killed cancer cells or treated with LPS and even returned to the levels close to those of naive DCs. In contrast, the level of IL-12 production in DCs co-incubated with immunogenically killed cancer cells was significantly higher than in naive DCs and similar to the IL-12 level 24 h after co-incubation (*Figure 6* and *Figure 7*).

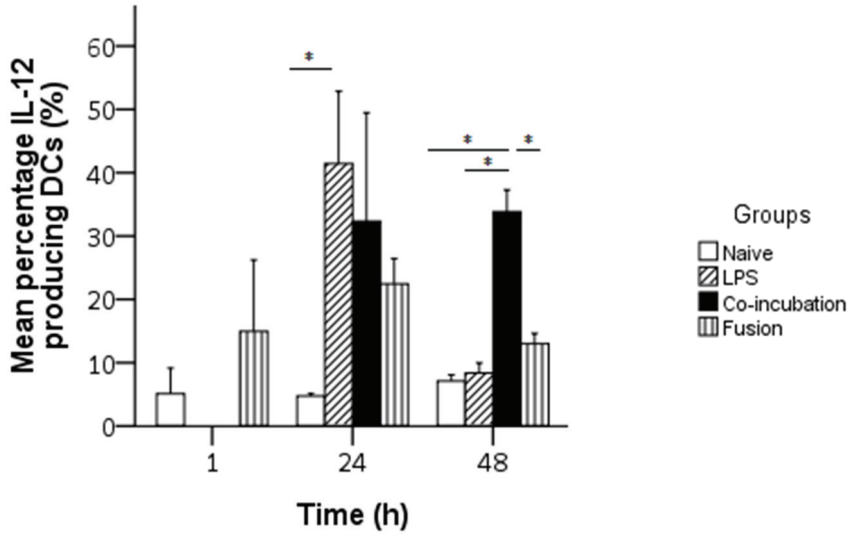

FIGURE 6. Characterization of CC-DC hybrids. Influence of fusion or co-incubation of EO771 cells with DCs on functional DC maturation (IL-12 production) (n= 3, error bars ± 1 SD).

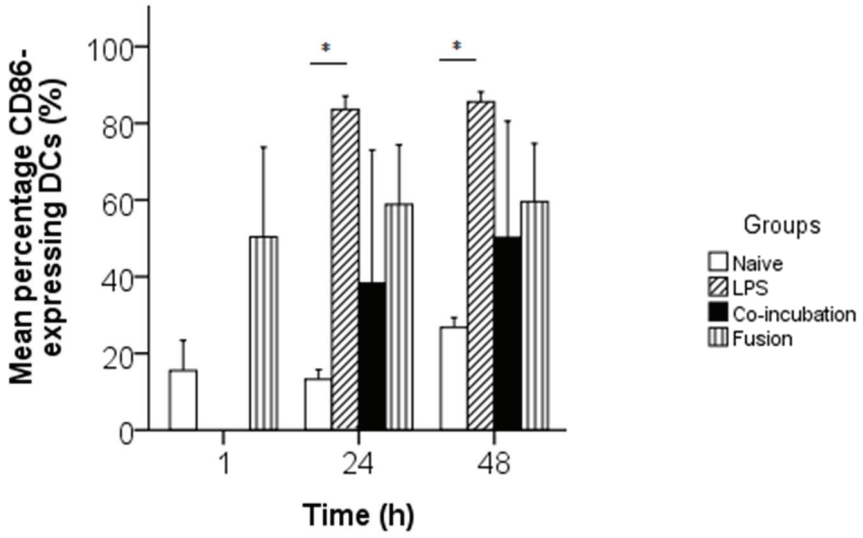

FIGURE 7. Characterization of CC-DC hybrids. Influence of fusion or co-incubation of EO771 cells with DCs on phenotypical DC maturation (CD86 surface expression) (n= 3, error bars ± 1 SD).

***In vivo* effects of whole cancer cell vaccines based on immunogenically killed cancer cells followed by tumor inoculation.** Three groups of 11 mice were vaccinated with either immunogenically killed EO771 cells (cancer cell vaccine group), immunogenically killed EO771 cells co-incubated with DCs (co-incubation vaccine group) or fused with DCs (fusion vaccine group). A fourth group of 11 mice served as control and was treated with a saline solution (negative control group). The vaccination and sample collection schedule is shown in *Figure 8*. The effect of immunogenically killed EO771 cells and co-incubation or fusion of immunogenically killed EO771 cells with DCs on the elicited immune response in mice was evaluated.

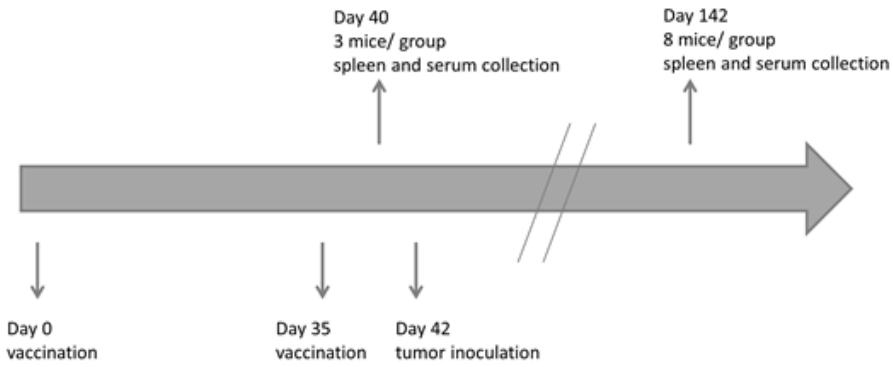

FIGURE 8. In vivo *experimental setup. All mice received on days 0 and 35 in the flanks (left and right flank, respectively) a subcutaneous injection of phosphate buffered saline (control group) or 1 of the 3 vaccines (cancer cell vaccine, co-incubation vaccine or fusion vaccine). Three mice in each group were euthanized on day 40 and spleen and serum were collected to characterize the cellular and humoral response (tumor-specific cytotoxicity, activation of Th1 or Th2 effector T cells, production of tumor-specific antibodies). Tumor inoculation was performed on day 42. Eight mice in each group were euthanized on day 142 (or earlier if the tumor would reach 1 cm²) and spleen and serum were collected for the characterization of the memory cellular and humoral response (tumor-specific cytotoxicity, activation of Th1 or Th2 memory T cells, production of tumor-specific antibodies).*

Five days after the second vaccination, cellular and humoral responses were evaluated in the mice of all 4 groups. The percentages of activated tumor specific T helper (Th)2 $CD4^+$ and $CD8^+$ T cells were significantly higher in the co-incubation vaccine group

than in the control group and the other vaccine groups. The latter vaccine groups did not generate more activated Th2 T cells than the control group (*Figure 9*).

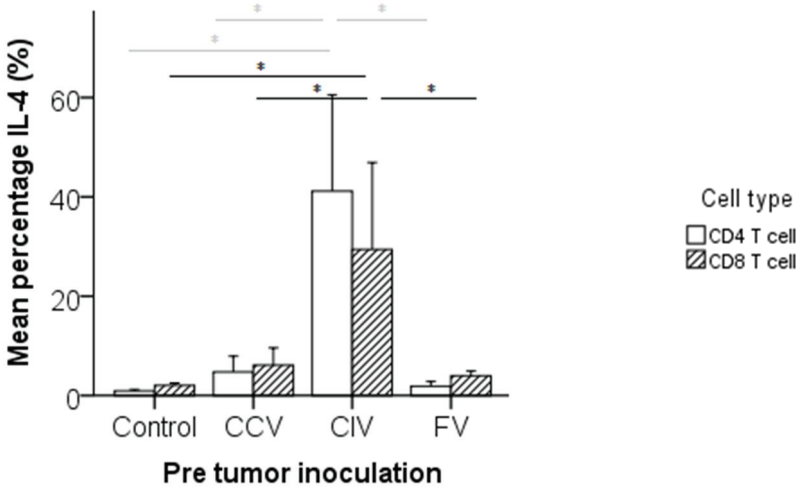

FIGURE 9. In vivo *evaluation of vaccines based on immunogenically killed EO771 cells. Activation of Th2 effector cells (IL-4 positive CD4+ and CD8+ T cells) 5 days after the second vaccination (n=3, ± 1 SD). Abbreviations: Control, control group; CCV, cancer cell vaccine group; CIV, co-incubation vaccine group; FV, fusion vaccine group; Pre, 2 days prior to tumor inoculation or 5 days after the second vaccination.*

The percentages of activated tumor specific Th1 or cytotoxic T lymphocyte (CTL) $CD4^+$ and $CD8^+$ T cells in the vaccinated groups were not significantly higher than in the control group. The cellular response was also characterized by measuring tumor-specific cytotoxicity (*in vitro* cancer cell killing capacity of isolated splenocytes). Splenocytes from the cancer cell vaccine group resulted in a significantly higher cancer cell killing than splenocytes collected from the fusion vaccine or control group (*Figure 10*), while those of the co-incubation vaccine group only differed significantly from the control group.

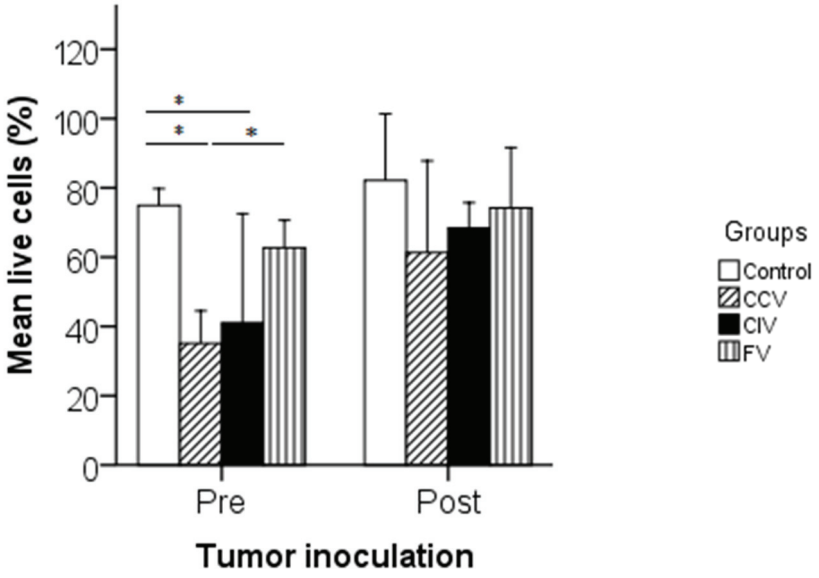

FIGURE 10. In vivo *evaluation of vaccines based on immunogenically killed EO771 cells. The humoral response (presence of antibodies against EO771 cells) (Pre n=3, Post n=8, ± 1 SD). Abbreviations: Control, control group; CCV, cancer cell vaccine group; CIV, co-incubation vaccine group; FV, fusion vaccine group; Pre, 2 days prior to tumor inoculation or 5 days after the second vaccination; Post, 100 days post tumor inoculation or 102 days after the second vaccination.*

Five days after the second vaccination, the humoral response (presence of antibodies against EO771 cells) was confirmed in all vaccinated groups, yet only the co-incubation and fusion vaccine group had, compared to the control group, significantly higher levels of IgG antibody directed against the EO771 cells. The highest antibody levels were measured in the co-incubation vaccine group, which parallels the high percentage of activated Th2 T cells in this group (*Figure 11*).

Six of 8 mice in the control group developed a tumor after the tumor inoculation, which differed significantly from 1 in 8 mice in the cancer cell vaccine group (p= 0.041) and none in the co-incubation and fusion vaccine group (p= 0.015).

Hundred days after the tumor challenge, significant differences in the type of immune response were observed between the different treatment groups at the time of necropsy. The percentages of activated tumor specific T helper (Th)2 $CD4^+$ and $CD8^+$ T cells in any vaccine group were not significantly higher than in the control group. The percentages of activated tumor specific Th1 T cells and CTL in mice of the cancer cell and the co-incubation vaccine groups were significantly higher than in the control group (*Figure 12*), indicating a clear memory T cell response.

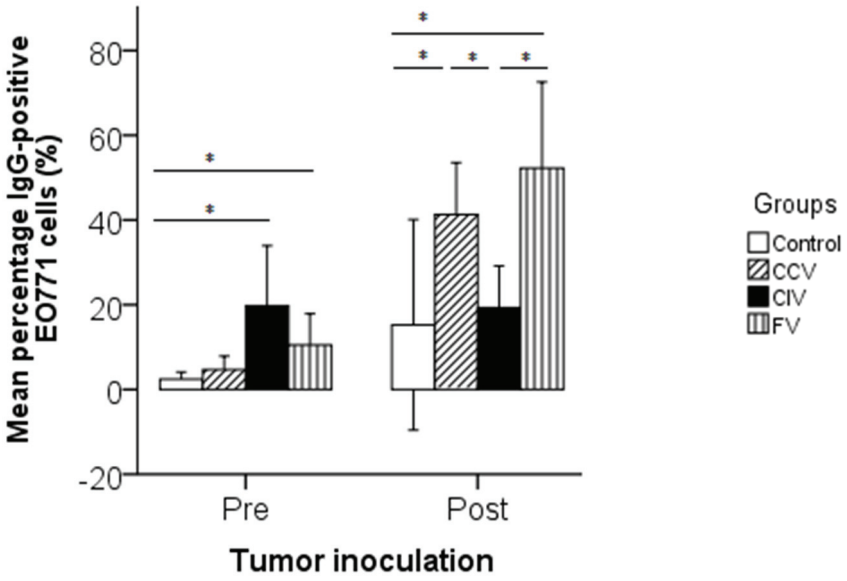

FIGURE 11. In vivo *evaluation of vaccines based on immunogenically killed EO771 cells. The cellular response for tumor-specific cytotoxicity (*in vitro *cancer cell killing capacity of isolated splenocytes. In this cytotoxicity assay, the percentage of live EO771 cells inversely correlates with the efficacy of the vaccine/group (Pre n=3, Post n=8, ± 1 SD). Abbreviations: Control, control group; CCV, cancer cell vaccine group; CIV, co-incubation vaccine group; FV, fusion vaccine group; Pre, 2 days prior to tumor inoculation or 5 days after the second vaccination; Post, 100 days post tumor inoculation or 102 days after the second vaccination.*

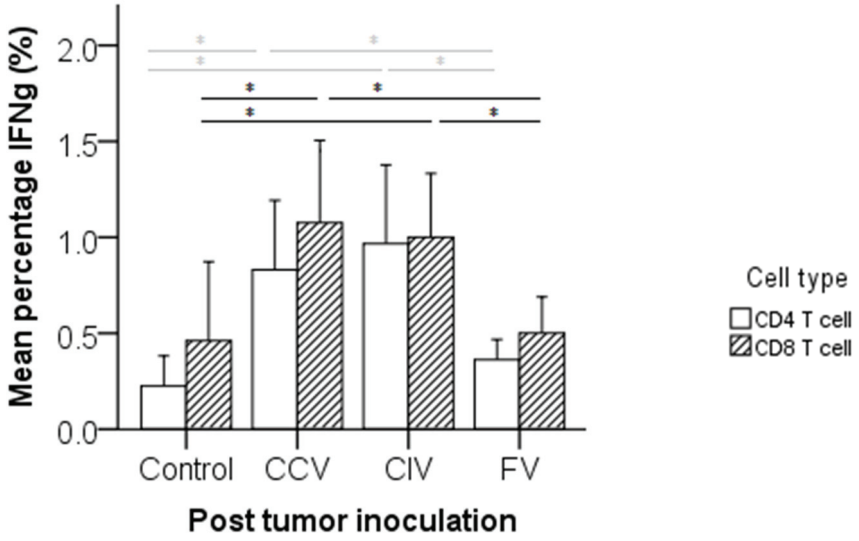

FIGURE 12. In vivo *evaluation of vaccines based on immunogenically killed EO771 cells. Activation of Th1 and CTL effector cells (IFNγ-positive CD4+ and CD8+ T cells) 102 days after the second vaccination (n=8, ± 1 SD). Abbreviations: Control, control group; CCV, cancer cell vaccine group; CIV, co-incubation vaccine group; FV, fusion vaccine group; Post, 100 days post tumor inoculation or 102 days after the second vaccination.*

The tumor-specific cytotoxic effect of the isolated splenocytes was also higher in these groups than in the control group, but did just not reach the significance level (p=0.064 and 0.085 respectively) (*Figure 10*). On the other hand, no indications of a cellular memory could be found in the fusion vaccine group. Hundred days after the tumor challenge, the level of tumor-specific antibodies was, compared to the control group, significantly higher in the cancer cell and fusion vaccine groups, but not in the co-incubation group (*Figure 11*). Thus, a clear humoral memory was established in the cancer cell and fusion vaccine group, but not in the co-incubation vaccine group.

5 Discussion

Association of cancer cells with DCs and ICD of cancer cells both improve the immunogenicity of whole cancer cell vaccines. We examined whether the immunogenicity of immunogenically killed cancer cells could be further enhanced through association with DCs. The impact of these constructs was monitored by measuring cellular and humoral (memory) responses of all included mice groups (*Table 9*).

TABLE 9. Cellular and humoral responses in the different vaccine groups

Vaccine groups	Cellular						Humoral	
	IFNγ (%)		IL-4 (%)		Cytotoxicity (%)		IgG (%)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Control	0.97 ± 0.28	0.46 ± 0.41	2.03 ± 0.47	16.26 ± 32.75	0.75 ± 0.05	0.82 ± 0.19	2.43 ± 1.66	15.26 ± 24.87
CCV	0.58 ± 0.23	**1.08 ± 0.43**	6.12 ± 3.47	4.96 ± 4.92	**0.35 ± 0.10**	0.61 ± 0.26	4.67 ± 3.23	**41.32 ± 12.22**
CIV	1.19 ± 0.22	**1.00 ± 0.33**	**29.42 ± 17.49**	2.22 ± 0.43	**0.41 ± 0.31**	0.68 ± 0.07	**19.79 ± 14.13**	19.34 ± 9.83
FV	0.87 ± 0.16	0.50 ± 0.19	3.99 ± 0.96	1.50 ± 0.78	0.63 ± 0.08	0.74 ± 0.17	**10.53 ± 7.35**	**52.22 ± 20.35**

Control: *control group;* ***CCV:*** *cancer cell vaccine group;* ***CIV:*** *co-incubation vaccine group;* ***FV:*** *fusion vaccine group;* ***Pre:*** *2 days prior to tumor inoculation or 5 days after the second vaccination;* ***Post:*** *100 days post tumor inoculation or 102 days after the second vaccination;* ***IFNγ:*** *IFNγ produced by CD4+ and CD8+ T cells stimulated with EO771 cell lysate (marker for CTL and Th1 activation);* ***IL-4:*** *IL-4 produced by CD4+ and CD8+ T cells stimulated with EO771 cell lysate (marker for Th2 activation);* ***Cytotoxicity:*** *in vitro efficacy of isolated splenocytes to kill EO771 cells;* ***IgG:*** *presence of IgG-antibodies on EO771 cells. Mean data with 1x standard deviation per group are represented, significant results are marked in bold.*

Although cancer cells used in whole cancer cell vaccines are often irradiated prior to use[299, 305, 306] and irradiation is able to cause ICD,[50] we cannot assume the antitumoral

effects of these vaccines arise from immunogenically killed cancer cells. Indeed, the question remains whether irradiation can specifically and efficiently induce ICD.[307] To unambiguously evaluate the effects of ICD, MTX, a well-known ICD inducing agent, was used as the ICD inducing stimulus.

None of the mice vaccinated with immunogenically killed cancer cells fused (fusion vaccine) or co-incubated (co-incubation vaccine) with DCs developed a tumor after tumor challenge. In the group that received only the immunogenically killed cancer cells one mouse developed a tumor after tumor challenge. In contrast, 75% of the mice of the control group did develop a tumor after tumor challenge (*Figure 13*).

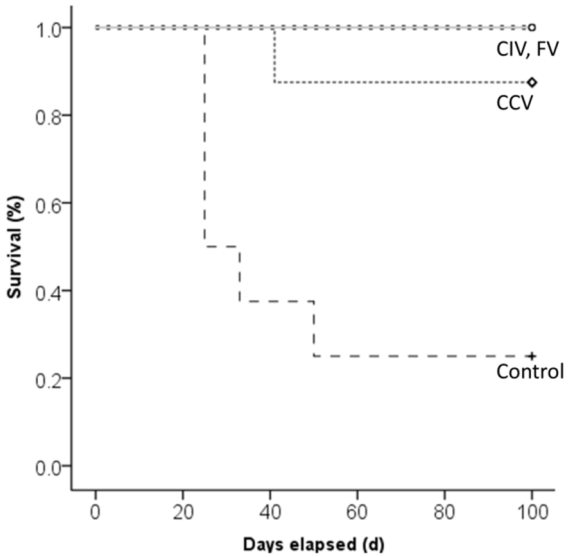

FIGURE 13. Kaplan–Meier survival curve after tumor challenge of prophylactically vaccinated mice (n=8/group). Abbreviations: CCV, cancer cell vaccine group; CIV, co-incubation vaccine group; Control, control group; FV, fusion vaccine group.

Therefore, it is sound to conclude that all tested vaccines induced a protective effect against cancer growth. Although all vaccinated groups were protected against tumor

development, our experiments indicated that the immunological responses accountable for this protection were different between the different vaccine groups.

In the co-incubation vaccine group, tumor-specific cytotoxicity and humoral responses were present shortly after the second vaccination. Surprisingly, hundred days later, a significant cellular memory response for CTL and Th1 activation was present but only a trend towards increased tumor-specific cytotoxicity and humoral response was observed. This decline in cellular and humoral response in time could potentially be explained by the fact that the co-incubation vaccine induced such a powerful effector effect that the responding lymphocytes were driven into terminal differentiation and did not have the opportunity to develop a memory response.[137]

In the fusion vaccine group, no cellular response was induced after vaccination and the observed humoral response appeared relatively weak. Hundred days after the tumor challenge, the humoral memory response demonstrated a 5-fold increase compared to the response before challenge, while a cellular response remained absent. The fact that none of the mice in this group developed a tumor suggests that a sole humoral response has protective effects against tumor development. The observed protection is potentially mediated through Th2-cytokine IL-24.[308] Indeed, although Th2 cells have been associated in the past with a contribution to tumor growth,[309] large numbers of tumor-infiltrating Th2 cells are associated with significantly improved disease-free survival in human patients with classical Hodgkin lymphoma.[310]

Vaccination with immunogenically killed cancer cells only induced a strong tumor-specific cytotoxicity response, but no humoral response. After the tumor challenge, there was a significant humoral and cellular memory response (for CTL and Th1 activation and a trend for tumor-specific cytotoxicity).

It is noteworthy that the initial cellular immune responses in the vaccinated groups seem to originate from CTLs with lytic activity that secrete no or low amounts of IFNγ, as we did not observe significantly higher amounts of IFNγ positive cells within 5 days after the second vaccination compared to the control group. Likewise, CTLs with lytic activity that do not secrete IFNγ have been reported by several other research groups.[311, 312] Indeed, IFNγ and cytotoxicity are regulated independently in $CD8^+$ T cells.[311]

Many previous studies with CC-DC fusion vaccines mentioned an overnight incubation of the fusion mixture.[105, 306, 313-316] Yet our *in vitro* data revealed that during overnight incubation of the fusion mixture, CC-DC hybrids are also formed via phagocytosis of unfused cancer cells by unfused DCs. Therefore, it is very likely that in overnight incubated fusion mixtures, two types of CC-DC hybrids are present: on the one hand hybrids that are formed via fusion, on the other hand hybrids that occur after phagocytosis of cancer cells by DCs. In our study, the CC-DC hybrids were purified shortly after fusion and hence the formation of hybrids through phagocytosis was prohibited. In the literature, humoral as well as cellular responses have been reported after vaccination with "unpurified" CC-DC fusion vaccines.[317] Our data documented that the fusion vaccine mainly operated through a humoral response, suggesting that the cellular response induced by unpurified fusion vaccines with overnight incubation most likely originates from CC-DC hybrids formed via phagocytosis and not via fusion. The lower immunogenicity of our fusion vaccine compared with the other fusion vaccines reported in the literature could be accredited to the use of pure CC-DC fusion hybrids. However, there are two other possible reasons that may explain why our fusion vaccine did not elicit a significant cellular response. First, it has been reported that in some CC-DC fusion hybrids based on mature DCs, like in this study, the anti-inflammatory cytokine IL-10 is produced, which tempers the cellular immune response and skews it toward a Th2 response.[318] Second, O'Connor et al[319] described reversal of mature DCs to an immature state after fusion[319] that is less capable of homing toward the lymph node. Therefore, cellular responses might not be induced, as they are highly dose responsive,[120] whereas for antibody responses the minimal antigen threshold is relatively low.[119]

The cancer cell and the co-incubation vaccine group both did induce cellular as well as humoral immune responses. It has been shown that primarily the lymph node residing CD11c+ macrophages are essential for the cross-presentation of dead cell antigens.[320] However, a small contribution of migratory DCs, as present in the co-incubation group, cannot be ruled out[320] and might explain the differences in timing and intensity of the immune reactions observed between these two vaccine groups.

Although it has been reported that SC injection of EO771 cells near the fat pad of the fourth mammary gland into C57BL/6 mice resulted in tumor development in 97% of the inoculated mice,[321] gross tumor growth was only present in 75% of the non-vaccinated mice. Yet, the number of affected mice in the control group versus any of the vaccine groups was significantly different.

Since the fusion process can reverse the maturation state of DCs, preferably immature DCs should be harvested for vaccination with fusion-based vaccines.[319] Dendritic cells in this work were selected through their expression for CD11c in order to remove non-hybridized MTX-treated EO771 cells from the CC-DC mix. Since mature DCs express more CD11c than immature DCs,[322] mature DCs were more likely selected prior to fusion. Consequently, the obtained response in the fusion vaccine group is possibly lower than it could have been. A possible solution for fusion-based DC vaccines could be selection of DCs based on DC immaturity markers CCR1, CCR2 or CCR5[319] after negative selection of DCs.

6 Conclusion

In conclusion, based on our findings, we stipulate that vaccination with immunogenically killed cancer cells alone or with CC-DC hybrids obtained via co-incubation or fusion of immunogenically killed cancer cells with DCs, results in the protection of vaccinated mice against tumor development when challenged 5 days after the second vaccination. However, the immune responses responsible for this protection differ between the vaccine types. The immunogenically killed cancer cells without DCs protect the mice against tumor development via a cellular response only. The pre-inoculation protection in the fusion vaccine occurs mainly via a humoral response, whereas the co-incubation vaccine protects the mice via a cellular as well as a humoral immune response.

The current study indicates that vaccination with immunogenically killed cancer cells (with or without DCs) may be a useful prophylactic strategy for cancer growth. Based on the finding that a non DC-based cancer cell vaccine provided protection when using immunogenically killed cancer cells, the manufacture of cancer cell vaccines can

potentially be simplified as the production of DC-based vaccines is laborious, time-consuming and expensive. Further research should be performed to confirm the advantages of ICD in cancer cell vaccines. Additional cancer models should be tested to document whether other cancer types will reveal similar results.

CHAPTER 2

Interleukin 12 in cancer-bearing pet dogs

This chapter is based on

Intratumoral interleukin 12 gene therapy stimulates the immune system and decreases angiogenesis in dogs with spontaneous cancer. Cicchelero L, Denies S, Haers H, Vanderperren K, Stock E, Van Brantegem L, de Rooster H*, Sanders NN*

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1 Abstract

Interleukin 12 (IL-12) is a powerful immunostimulatory cytokine with a strong antitumoral activity. In this work, the immunological, anti-angiogenic and clinical effects of 3 consecutive intratumoral IL-12 electrogene therapy (EGT) treatments were evaluated in 9 dogs with spontaneous cancer. In all dogs, tumor biopsies and blood samples were taken prior, during and after the intratumoral IL-12 EGT (on day 1, 8, 35 and 1, 3, 8, 15, 35 respectively). An initial decrease in immune cells was followed by an increase above baseline 1-3 weeks after treatment initiation. Interestingly, the decrease in peripheral leukocytes 2 days after intratumoral IL-12 EGT coincided with erythema and tumor swelling. Transient increases of IL-12 and IFN-γ were measured in the serum and the tumor tissue, whereas IL-10 transiently increased only in the serum. The effect of intratumoral IL-12 EGT on the levels of IL-24 and vascular endothelial growth factor in the sera and tumor biopsies differed per dog. Via contrast-enhanced ultrasound (on day 1, 8 and 35), we demonstrated that intratumoral IL-12 EGT resulted in a significant decrease of the relative blood volume and blood flow speed in the tumor compared to baseline. Metastases were present in 2 dogs. In 1 of these dogs, IL-12 EGT of the primary tumor was associated with a transient partial regression of the metastases, but not of the primary tumor. The second dog with metastases did not survive long enough to complete the entire treatment cycle.
Despite encouraging immunostimulatory and anti-angiogenic effects after intratumoral IL-12 EGT, no clinically relevant outcomes were observed in this study, since persistent tumor regression could not be obtained. On the other hand, the laboratory and ultrasound results hold great promise for combinatorial strategies of intratumoral IL-12 EGT with conventional antitumor (immuno)therapies.

2 Introduction

During the last decennia, our knowledge on how cancer cells interact with the cancer patient's immune system has exponentially increased. This has resulted in the belief that immunotherapy can play an important role in the treatment of cancer. In

principle, the immune system is able to recognize and kill cancer cells. Yet the tumor and its microenvironment are capable of creating a protective immunosuppressive shield.[45] Unfortunately for the host, suppressor pathways in cancer immunology generally have a dominant effect over activating stimuli.[45] In this way, a general tolerance toward tumor antigens will be generated when tumor antigens are simultaneously presented by immune-stimulating and by tolerizing antigen presenting cells (APCs). Therefore, part of the treatment strategy in cancer patients should aim at tackling the protumoral microenvironment, allowing the immune system to exert its antitumoral effects.[45, 237]

Interleukin 12 (IL-12) is an immunostimulatory cytokine, produced by different immune cells like dendritic cells (DCs). It plays a key role in the regulation of inflammation by linking innate and adaptive immune responses. In addition, it has powerful antitumoral properties. Interleukin 12 leads to activation and proliferation of T cells and Natural Killer (NK) cells.[200, 201, 202] It is able to recruit NK cells, activated T cells and polymorphonuclear (PMN) cells,[198, 199] making IL-12 a useful booster of a pre-existing immune response. In turn, activated T cells produce interferon (IFN) γ, which induces a positive feedback loop on IL-12 secretion by APCs.[197] Not only can IL-12 engender instant activation of (antitumoral) effector cells, it can also program effector T cells for optimal generation of effector memory T cells and T follicular helper cells.[203, 204] Furthermore, IL-12 decreases the suppressive function of myeloid-derived suppressor cells (MDSC)[207] and inhibits the differentiation of T cells to regulatory T cells (Tregs) as well as the expansion of Tregs.[205, 206] An additional antitumoral mechanism of IL-12 is the inhibition of angiogenesis through the induction of IFNγ-Inducible Protein (IP) 10 and Monokine Induced by Gamma IFN (MIG). These two cascade products of IFNγ inhibit the formation of new blood vessels[208] by i.a. decreasing the production of angiogenic factors such as vascular endothelial growth factor (VEGF) and metalloproteinase (MMP) 9.[208, 209, 210, 211, 212]

Interleukin 12 has intriguing antitumoral features; yet grave toxicity has been shown when recombinant IL-12 protein was administered intravenously (IV) to human cancer patients.[213] This toxicity has urged the need to find alternatives for systemic IL-12 treatment and led to the evaluation of tumor-specific delivery methods of IL-12. A

simple and often used approach to target IL-12 to the tumor consists out of intratumoral IL-12 gene therapy. This can be accomplished through various ways and one of which is electroporation, a reproducible and highly efficient method to deliver plasmid (p)DNA.[214] Electroporation is the short administration of electrical pulses to ensure the formation of transient pores into the plasma membrane of cells, thereby facilitating the intracellular delivery of large molecules such as pDNA.[215] Once the pDNA reaches the cell's nucleus, the production of the protein encoded by the plasmid ensues.[216, 217] *In vivo* electroporation does not cause any severe adverse events,[218] and several canine and human clinical trials showed the safety and clinical efficacy of electroporation-mediated intratumoral delivery of a pDNA encoding IL-12.[235, 219, 220, 221] Those studies present evidence that it is possible to overcome local suppressor pathways in tumors and tip the balance in favor of an antitumor immune response. Regression of electroporated primary tumors[223, 221, 146, 235] and distant non-treated metastases[219, 222, 223] has been observed when small tumors of human and canine cancer patients were treated with intratumoral IL-12 gene therapy. However, in these studies successes were present in only a subset of patients, whereas others patients seemed refractory to such approach.[146, 219, 221, 222]

Given the above, we wanted to investigate whether the effects of IL-12 therapy on the immune system are comparable in every dog with spontaneous cancer. To this end, we aimed to examine in more detail the immunological and anti-angiogenic effects of intratumoral IL-12 EGT in canine cancer patients with different tumor types by serial tissue biopsies and blood samples. The immunological effects were studied by monitoring the blood levels of lymphocytes, monocytes and granulocytes. Also the serum and intratumoral levels of key Th1 and Th2 cytokines were studied. The anti-angiogenic effect of the IL-12 gene therapy treatment was studied by monitoring local and systemic VEGF levels as well as by contrast-enhanced ultrasound. Contrast-enhanced ultrasound is a technique that has recently been validated to measure the efficacy of anti-angiogenic treatments in clinical trials.[323] To detect possible side effects of IL-12 gene therapy, we monitored the dogs by regular physical examinations and by analyses of selected hematology and biochemistry parameters.

3 Materials and methods

3.1 Animal selection

Client-owned dogs with spontaneous malignant neoplasms were enrolled at the Faculty of Veterinary Medicine of Ghent University between October 2014 and April 2015 if they met the following inclusion criteria: a histologically confirmed neoplasia accessible for application of electrodes, normal cardiovascular function, good general health status and a biochemistry profile within reference range. Patients included in the study were not eligible for surgery, either due to recurrent disease for which conventional surgery was already exhausted, or due to impractical location of the tumor. Baseline bloodwork consisted of a complete blood count (CBC) with differential white blood cell count, which was performed using an automated laser hematology analyzer (Procyte, Idexx, The Netherlands) with species-specific software (Vetlab Station). The automated chemistry analyzer Technicon RA-XT (Catalyst, Idexx, The Netherlands) was used for the determination of the following biochemical parameters: blood urea nitrogen (BUN), creatinine, serum alkaline phosphatase (SAP), alanine aminotransferase (ALT), glucose, total protein, and globulins.

The study cohort consisted of 9 dogs, 5 castrated males and 4 spayed females of 8 different breeds (Golden Retrievers (n=2), Malinois, Bouvier, Maltese, Labrador Retriever, Bichon Frisé, French Bulldog and Bordeaux Dog); their age ranging from 2.9 to 13.7 years (mean 9.5 ± 4.1years) (*Table 10*).

In all animals, screening for metastatic disease was accomplished by thoracic radiographs and/or computer tomography (CT) and/or abdominal ultrasonography. Other conventional therapies such as chemotherapy or radiation were not given prior to intratumoral hIL-12 EGT, except in dog 3 (radiotherapy and metronomic chemotherapy were discontinued 6 weeks and 1 month respectively prior to study entry) and dog 8 (chemotherapy was discontinued 2 weeks prior to study entry). Animals were free of non-steroidal anti-inflammatory drugs (NSAID) at least 1 week before and during the entire study period. All animal handling and treatment

procedures were approved by the Ethical Committee (approval number 2013/166) of the Faculty of Veterinary Medicine of Ghent University, Belgium.

TABLE 10. Signalment, tumor type, tumor size, presence of metastases and IL-12 EGT treatment regimen for each dog

Dog	Breed	Sex	Age	Tumor type (localization)	Metastases	Treatment frequency	Tumor size
1	Golden Retriever	Mn	13y 7m	Schwannoma (dorsal to carpus)	Regional lymph node cytology and thoracic X-rays negative	Day 1, 3, 5	314 cm^3
2	Golden Retriever	Mn	5y 10m	Fibrosarcoma (rostromaxillar)	Regional lymph node cytology and thoracic X-rays negative	Day 1, 3, 5	2.9 cm^3
3	Bichon Frisé°	Mn	2y 11m	Osteosarcoma (left maxillary, zygomatic arch and bone, sphenoid and ventral part of the nasal conchae)	Regional lymph node cytology and thoracic CT negative	Day 1, 3, 5*	Extensive skull invasion, approx. 13%
4	Malinois	Fn	6y 9m	Tubulopapillar complex carcinoma (mammary glands)	Regional lymph node cytology positive, thoracic X-rays negative	Day 1, 8, 15	58.6 cm^3 1.8 cm^3 7.1 cm^3 15.7 cm^3
5	Bouvier	Fn	12y 9m	Fibrosarcoma (right maxillary, palatine and sphenopalatine bone, and invasion of the nasopharynx)	Regional lymph node cytology and thoracic CT negative	Day 1, 8, 15	Extensive skull invasion, approx. 15%
6	Maltese	Fn	12y 7m	Adenocarcinoma (perianal)	Regional lymph nodes ultrasound positive	Day 1, 8, 15	10.2 cm^3

7	Labrador Retriever	Mn	11y 9m	Schwannoma (dorsal to carpus)	Regional lymph node cytology and thoracic X-rays negative	Day 1, 8, 15	12.5 cm^3
8	French Bulldog°	Mn	13y 7m	Mastocytoma (interocular)	Regional lymph node cytology and thoracic X-rays negative	Day 1, 8*	25.1 cm^3 and 2.4 cm^3
9	Bordeaux Dog	Fn	6y 1m	Squameus Cell Carcinoma (right maxillary)	Regional lymph node histopathology positive, thoracic X-rays negative	Day 1, 8*	75.4 cm^3

F: *female;* ***Fn:*** *female neutered;* ***M:*** *male;* ***Mn:*** *male neutered.*
** patient did not survive long enough to finish the entire treatment cycle*
° patient received chemotherapy or radiation therapy prior to IL-12 EGT

3.2 Plasmid

An IL-12 plasmid encoding human IL-12 (hIL-12) was obtained via Celsion-EGEN, Inc. (Huntsville, AL, USA). It was produced under GMP conditions by Eurogentec (Seraing, Belgium). The hIL-12 plasmid contains consecutively an immediate early enhancer and a promoter derived from cytomegalovirus (CMV), a 5' untranslated region (UTR), a synthetic intron, the human p35 gene, the human growth hormone (hGH) 3' UTR and polyadenylation signal sequence, a CMV promoter, a 5' UTR, a synthetic intron, the human p40 gene, the hGH 3' UTR and polyadenylation signal sequence. Of note is the fact that both the hIL-12 subunits are individually expressed under the control of 2 separate CMV promoters. The sequences of the p35 and p40 cDNAs are identical to the sequences found in GenBank with the exception of a single base mutation in the second codon of the p35 cDNA. The change was made to insert a KOZAK sequence for proper translation initiation and also created an Nco I restriction digest site. The mutation changed the second amino acid of the p35 signal sequence

from a cytosine (CYS) to a glycine (GLY). A similar change was made to the second codon of the p40 cDNA.

3.3 Ultrasound and contrast-enhanced ultrasound

Prior to lL-12 treatment, B-mode ultrasound (US) of all tumors was performed with a linear 12-5 MHz or 17-5 MHz transducer (Philips, iU22 xMATRIX, Philips Medical systems, Bothell, Washington, USA) to determine the echogenicity, to assess the presence of mineralization within the tumor and to measure the tumor dimensions.

Following conventional US examination, tumor perfusion measurements with contrast-enhanced ultrasound (CEUS) were performed with a dedicated machine (Philips, iU22 xMATRIX, Philips Medical systems, Bothell, Washington, USA) equipped with contrast-specific imaging technology using a linear 12-5 MHz probe. To evaluate the tumor perfusion, dogs were imaged with manual restraint or, in 1 dog, under mild sedation with butorphanol (Dolorex® 0.2 mg/kg), a drug that is known not to influence the CEUS.[324] Prior to injection of microbubbles (MB), several imaging parameters were adjusted to decrease early destruction of the injected MBs by the US beam. Mechanical index was decreased (0.09), persistence was switched off and a single focal spot was placed under the tumor.

MB contrast agent (Sonovue®, Bracco, Milan, Italy), consisting of lipid-stabilized MBs with a sulfur hexafluoride gas core, was injected intravenously as a bolus (0.04 mL/kg), immediately followed by a 1 mL flush of sterile saline (Mini-Plasco NaCl 0.9%, Braun) via a peripheral venous catheter inserted in the cephalic vein. Between the boluses, the remaining MBs were destructed by increasing the acoustic power in the imaging plane for several minutes.

During CEUS, areas that should be avoided during injection of the IL-12 pDNA solution, such as main tumor vessels or necrotic tissue, were identified. All CEUS studies were digitally registered as a movie clip at a rate of 10 frames/sec for 90 sec after bolus injection. These clips were analyzed using integrated dedicated specialized computer software (VUEBOX, Bracco) for objective quantitative analysis. Tumor regions-of-interest (ROIs) were manually drawn and mean pixel intensities and time-

intensity curves created per ROI. These curves were analyzed for Radius Basis Function parameters representing blood volume (area-under-curve) and blood velocity (wash-in rate). A B-mode image was projected simultaneously with CEUS imaging (*Figure 19*), and (CE)US was repeated for comparison at the same tumor location at day 8 and 35.

3.4 Electrogene therapy

One mg of IL-12 plasmid per session was diluted with calcium and magnesium free phosphate-buffered saline (DPBS) (14040-174, Invitrogen). The injection volume was 1/6 of the tumor volume that was calculated with the following formula: L*H*Wπ/6 (L: length, H: height, W: width). The injection volume was administered intratumorally throughout the whole tumor mass. An 8-needle array electrode connected to an electroporator (Agile Pulse generator, BTX® Harvard Apparatus) was inserted within 2 min after injection around the injection site. Two pulses of 450 V/cm were given (pulse duration was 0.05 msec and the interval between the pulses 0.2 msec). After 50 msec, those 2 pulses were followed by 8 pulses of 100 V/cm (pulse duration was 10 msec and the interval between pulses 20 msec).

3.5 Clinical trial design

A written informed consent was obtained from each dog owner. Prior to each treatment session, the dog owner was interviewed and each dog was physically examined. Blood was collected to assess the patient's hematology and biochemistry. When all values were within reference range, a peripheral venous catheter was placed. Hair overlying tumor nodules was clipped prior to (CE)US. After a general US examination of the region of the tumor, the patients received IV contrast agent for CEUS evaluation of tumor perfusion. Afterward, the subject was anesthesized for the electroporation of the hIL-12 pDNA. For this purpose, the dog was premedicated with IV buprenorphine (Vetergesic® 10 µg/kg) followed 15 min later by IV dexmedetomidine (Dexdomitor® 5 µg/kg). After another 15 min, induction with IV propofol (Propovet multidose® 1 mg/kg) followed and was, if necessary, maintained with isoflurane.

A sterile IL-12 pDNA solution was injected directly into the tumoral tissue. The tumor volume treated was restricted by the needle length of the electrodes. Since the maximum length of the electrodes was 25 mm, tumor tissue at a depth greater than 25 mm could not be electroporated. After electroporation of the IL-12 pDNA into the tumor, 3 punch biopsies of 2 mm (Bap Medical, 33-31) were taken from different locations within the neoplasm to factor in intratumoral heterogeneity;[325] biopsies were taken after electroporation to avoid leakage of the diluted pDNA from the tumor. After the biopsy taking, the subjects were antidoted with IM atipamezole (Revertor®, 25 µg/kg). No (N)SAIDs were administered, to avoid interference with the development of an inflammatory response to the treatment. The non-anti-inflammatory pain killer Tramadol (Tramadol EG®, 2 mg/kg) was offered per os as alternative.

The occurrence of local side effects was evaluated at each follow-up with physical examination of the patient and careful assessment of the appearance of the electroporated area for signs of local inflammation (erythema, swelling, pain), secretions, necrosis, etc. Additionally, the occurrence of systemic effects was evaluated by measuring the amount of IL-12, IFNγ, IL-10, VEGF and IL-24 in serum and by screening for aberrations in CBC and/or the biochemistry profile. The response to therapy was assessed with repeated measurements of tumor size and perfusion through (CE)US and measurement of the amount of IL-12, IFNγ, IL-10, VEGF and IL-24 in tumor tissue.

The first 3 dogs (dogs 1, 2 and 3) were treated with a 1-day interval between treatments, but 1 of these dogs developed transient immune-mediated anemia and another fatal thrombocytopenia. Since both side effects could be attributed to IL-12 toxicity,[146] the treatment regimen was altered to a 1-week interval between treatments for all following patients (*Figure 14*).

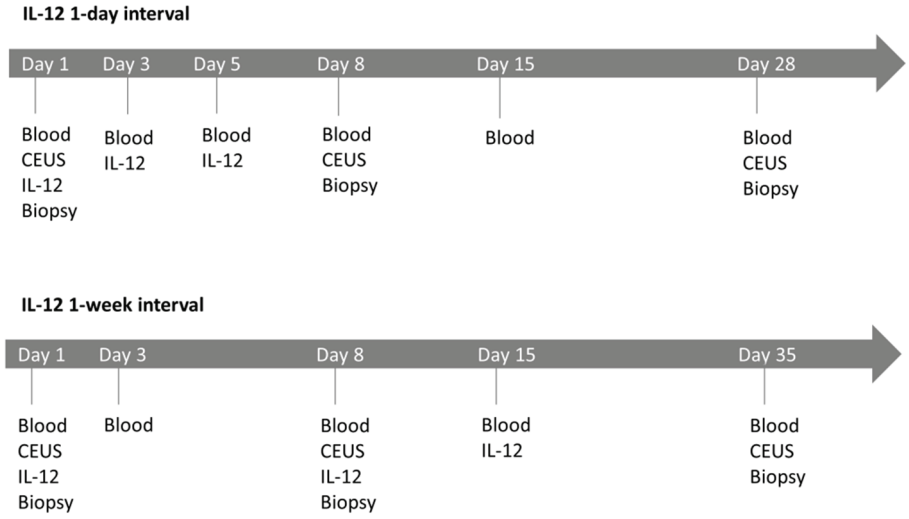


FIGURE 14. Initial hIL-12 EGT regimen (top) and adapted regimen (bottom) after the observation of severe side effects in 2 of the 3 patients treated with the initial regimen. Blood: complete blood count and biochemistry analysis, CEUS: contrast-enhanced ultrasound, IL-12: intratumoral injection of 1 mg hIL-12 plasmid DNA and electroporation, Biopsy: collection of 3 small tumor biopsy samples.

3.6 Tumor tissue analysis

The biopsies were immediately kept on dry ice and stored in a -150°C freezer (Panasonic, TCPS) until further use. A day prior to ELISA analysis biopsies were thawed in a 500 µL mixture of DPBS (Invitrogen) and protease-inhibitor cocktail (cOmplex Mini, Roche, 5892970001). Biopsy samples were disrupted with a tissue disruptor (TissueRuptor®, Qiagen, Germany) during 30 sec to lyse the cells. The lysed cells were centrifuged at 12,000xg during 10 min at 4°C. Afterward, the supernatant was removed and stored at 4°C until use the following day.

3.7 ELISA of local and systemic cytokines during intratumoral hIL-12 EGT

The presence of canine IFNγ, VEGF, IL-10 and IL-24 in serum and tumor tissue was determined using ELISA kits (Canine IFN-γ/VEGF/IL-10 Duoset ELISA Kit, R&D Systems, DY781B/DY1603/DY735, USA and Interleukin 24 ELISA Kit, MyBiosource, ABIN2088813 respectively) and human IL-12 with human IL-12 (p70) ELISA MAXTM Standard (Biolegend, 431701). In order to assess if the intratumoral IL-12 EGT was successful, the amount of human IL-12 in tumor tissue was compared for each dog on day 1, 8 and 35 of the treatment. The stimulatory effects of human IL-12 were determined by analysis of the amount of canine IFNγ, anti-angiogenic effects by VEGF analysis, anti-immunosuppressive effects by IL-10 analysis and antitumoral Th2 responses by IL-24 analysis. Intratumoral cytokines were assessed on day 1, 8 and 35, while the serum cytokine levels were measured on day 1, 3, 8, 15 and 35 for each dog. The Bradford test (Pierce™ Coomassie (Bradford) Protein Assay Kit, Invitrogen, 23200) was performed to take into account the differences in the amount of protein between samples.

3.8 Statistical Analysis

All data were analyzed with the statistical software program SPSS (version 19.0). The parametric data were analyzed with the paired sample t-test, the non-parametric data with the Wilcoxon rank-sum test with Bonferroni correction. Statistical significance was determined at $p \leq 0.05$. No statistical analysis could be performed on the ELISA data, since in some samples the cytokine levels were below the standard curve.

4 Results

4.1 Intratumoral and systemic release of cytokines in dogs that completed a whole IL-12 treatment cycle

To monitor the effects of IL-12 treatment, the levels of cytokines were analyzed at day 1 (baseline level), 8 and 35 in tumor biopsies and at day 1 (baseline level), 3, 8, 15 and 35 in blood samples. After intratumoral electroporation of the pDNA encoding hIL-12 (hIL-12 electrogene therapy (EGT)), a transient intratumoral increase of hIL-12 was observed in 4 out of the 6 dogs that completed the treatment regimen. One of the 2 dogs that did not have elevated hIL-12 levels in the tumor had increased hIL-12 levels in the serum. A clear, although transient, release of hIL-12 in the serum was only observed in 2 dogs (*Figure 15* and *Supplementary table 1*). Similarly, a transient increase of IFNγ in the tumor was measured in 1 out of 6 dogs and none of the dogs showed a clear increase of IFNγ in the serum (*Figure 15* and *Supplementary table 1*). The IL-12 treatment did not cause change of the IL-10 levels in the tumor, whereas mild increases in the serum were measured in 3 out of 6 dogs (*Figure 15* and *Supplementary table 1*). An intratumoral decrease of VEGF was measured in 2 out of 6 dogs; serum VEGF-levels remained unchanged in all 6 (*Supplementary table 1*). Due to technical issues, IL-24 measurements were only performed in 5 of the 6 dogs. An intratumoral decrease of IL-24 was measured in 3 out of 5 dogs, an increase in 1 dog and no change in the remaining dog. A serum increase of IL-24 was apparent in 5 out of 5 dogs, but started at different days (3 out of 5 at day 8, 1 out of 5 at day 15 and 1 out of 5 at day 35) (*Supplementary table 1*).

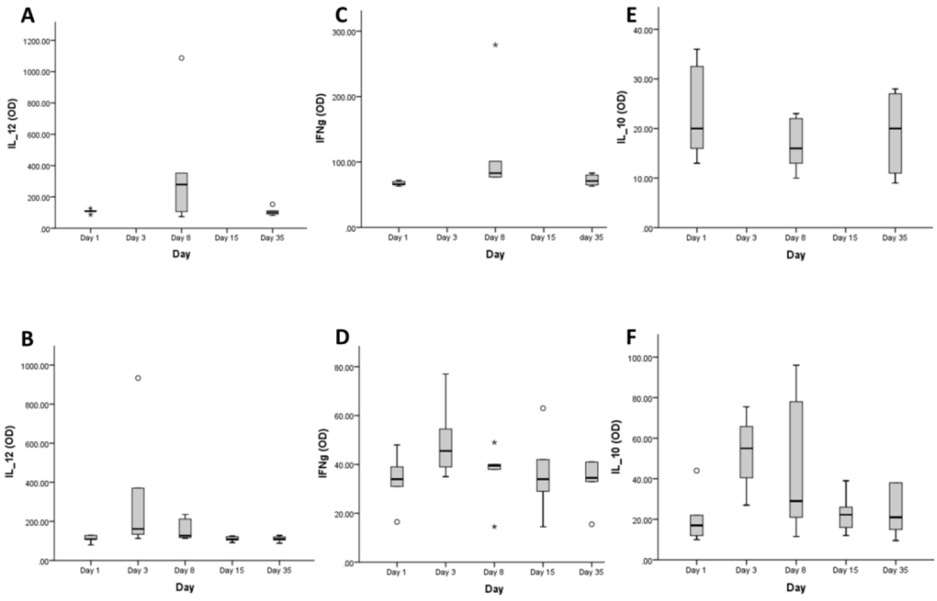


FIGURE 15. Intratumoral and serum absorbance values of IL-12 (A, B resp), IFNγ (C, D resp), IL-10 (E, F resp) prior, during and after intratumoral IL-12 EGT. The top rows represent the intratumoral values, the bottom rows the serum values. The Bradford test indicated similar amounts of protein between samples. Since several values were beneath the standard curve, significance could not be indicated and measurements are displayed in optical density (OD).

4.2 Complete blood count and biochemistry in dogs that completed a whole treatment cycle

After the first intratumoral hIL-12 EGT, a similar temporary decrease in peripheral leukocytes was observed in all 9 patients. Below, only the results of the 6 dogs that completed the whole treatment cycle are represented. The amount of lymphocytes, eosinophils and basophils initially decreased in each patient, but recovered above baseline in each patient as well (*Figure 16*). These effects were irrespective of their treatment regimen (*Supplementary figure 1*). A temporary increase in monocytes was observed between days 1-5 of treatment (*Figure 17* and *Supplementary figure 1*). The amount of red blood cells initially decreased in each patient (*Figure 16*), irrespective of

their treatment regimen (*Supplementary figure 1*). Subsequent intratumoral hIL-12 EGT did not further decrease the hematocrit. In contrast, a steady increase of hematocrit values was observed, and at day 35, the hematocrit values almost reached their baseline levels. Two out of 3 dogs in the early 1-day regimen experienced clinically relevant decreases in hematocrit (dog 1) or thrombocyte count (dog 3). The hematocrit of dog 1 recovered spontaneously, the thrombocytopenia of dog 3 did not despite 3 blood transfusions. No clinically relevant effects for hematology and biochemistry were observed in the remaining 6 dogs that were treated with the 1-week regimen.

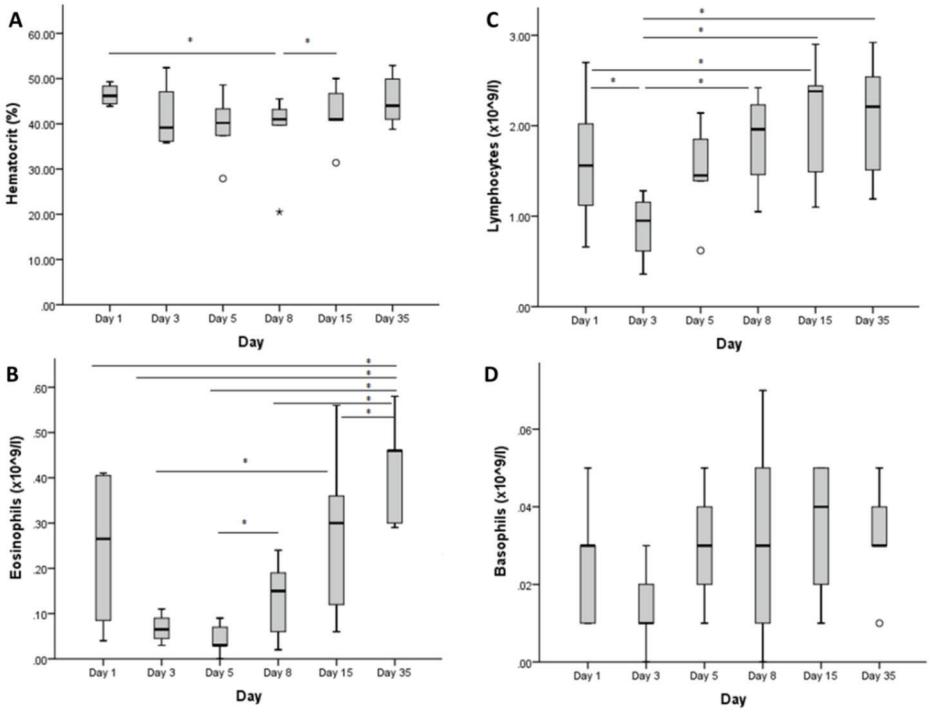


*FIGURE 16. Temporary decrease in hematocrit (day 1-3) (A) and amount of peripheral eosinophils (day 1-5) (B), lymphocytes (day 1-3) (C), and basophils (day 1-3) (D) following intratumoral hIL-12 EGT. *, p≤ 0.05 between groups.*

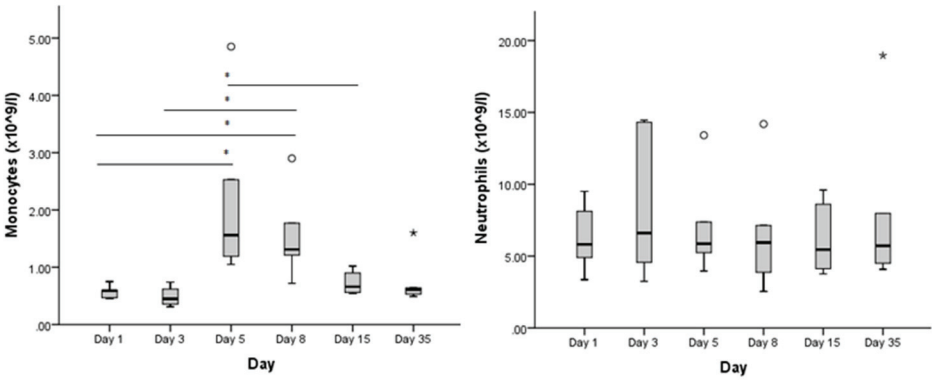


*FIGURE 17. Temporary increase in amount of peripheral monocytes (day 3-15) and neutrophils following intratumoral hIL-12 EGT. *, p≤ 0.05 between groups.*

All biochemical parameters remained within reference range and no significant changes were detected throughout the treatment cycle.

4.3 Physical examination findings and side effects after intratumoral hIL-12 EGT

During electroporation, clear muscle contractions occurred which required appropriate sedation. To assess possible side effects of the procedure, physical examinations (*Supplementary Data 1*), complete blood counts and biochemical parameters were performed at least on day 1, 3, 8, 15 and 35 of the treatment cycle. The VCOG-CTCAE criteria for adverse effects[326] associated with IL-12 toxicity were used to evaluate the findings of these examinations and are listed in *Table 11*. Generally, erythema and swelling were apparent 2-3 days after the first intratumoral hIL-12 EGT (*Figure 18*). Two dogs (dogs 1 and 3), treated with the 1-day regimen, developed systemic toxicities. Dog 1 developed immune-mediated anemia (Coomb's positive for IgG (H+L)) and dog 3 thrombocytopenia (the platelet amount was too low to establish an immune-mediated cause). Following adaptation of the regimen to a 1-week interval in the remaining 6 dogs, local or systemic side effects were not observed after intratumoral hIL-12 EGT in any of those patients.

TABLE 11. VCOG-CTCAE criteria for adverse effects[326] associated with IL-12 toxicity

Dog	Allergic / Immunological event	Blood/bone marrow			Constitutional clinical signs			Gastrointestinal			Tumor Pain
	Auto-immune disorder	Neutropenia	Neutrophilia	Thrombo-cytopenia	Fatigue	Fever	Weight loss	Anorexia	Diarrhea	Vomiting	
1	Grade 1 (possible)	-	-	-	1	-	1	1	-	-	1
2	-	-	-	-	-	-	-	-	-	-	-
3	No confirmation possible	-	-	5	1	2	1	-	-	-	-
4	-	-	-	-	1	1	-	1	-	-	1
5	-	-	-	-	1	-	1	2	1	-	1
6	-	-	-	-	1	-	1	1	-	-	1
7	-	-	-	-	1	-	-	-	-	-	1
8	-	-	-	-	2	-	3	2	-	-	1
9	-	-	-	-	3	-	1	2	-	-	1

Auto-immune disorder grade 1 indicates a mild auto-immune reaction. Thrombocytopenia grade 1 indicates mild thrombocytopenia; grade 5 indicates death as a consequence of thrombocytopenia. Fatigue grade 1 indicates mild lethargy; grade 5 indicates death as a consequence of lethargy. Fever grade 1 indicates 39.5–40.0° C; grade 5 indicates death as a consequence of fever. Weight loss grade 1 indicates <10% from baseline; grade 5 indicates death as a consequence of weight loss. Anorexia grade 1 indicates coaxing or dietary change required to maintain appetite; grade 5 indicates death as a consequence of anorexia. Diarrhea grade 1 indicates increase of up to 2 stools per day over baseline; grade 5 indicates death as a consequence of diarrhea. Tumor pain grade 1 indicates mild pain not interfering with function; grade 4 indicates disabling or uncontrollable pain.

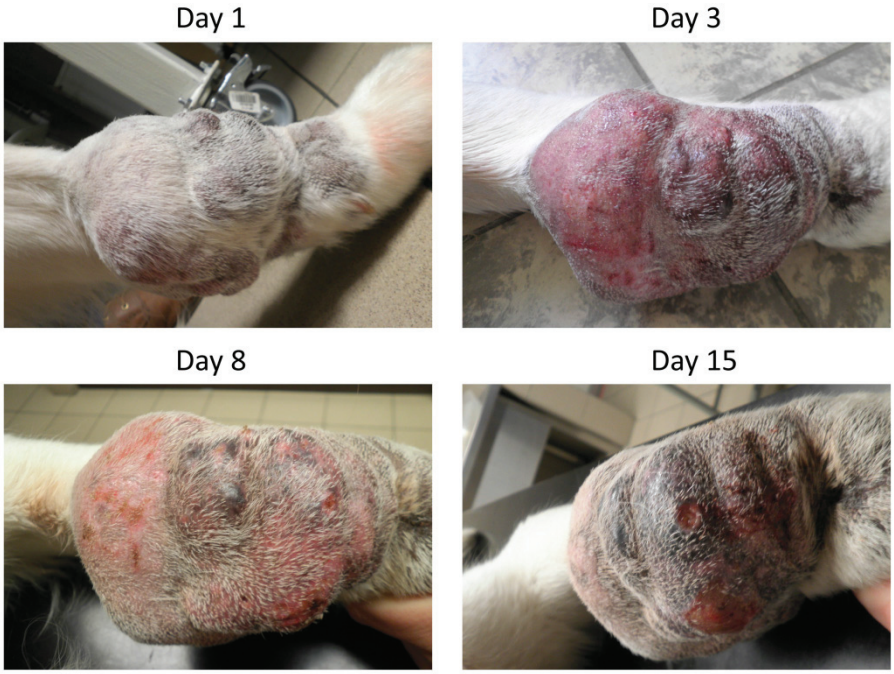


FIGURE 18. Representative tumor inflammation after intratumoral hIL-12 EGT in a schwannoma dorsal to the carpus (dog 1). The tumor inflammation is recognized by erythema on day 3 and swelling on day 8. Day 1: before treatment, day 3, 8, 15: after treatment initiation (1-day regimen).

4.4 Effects of intratumoral hIL-12 EGT on primary and metastatic tumor progression

Despite 3 intratumoral hIL-12 EGT treatments, all primary tumors continued to enlarge. Two of the included dogs (dog 6 and 9) had metastases before the start of the study. Dog 9 did not survive long enough to evaluate the anti-metastatic effects of the treatment. The abdominal metastases in the left and right medial iliac lymph nodes (LN) of dog 6 did show a transient response to treatment. The left medial iliac LN demonstrated a slow growth initially (size of 0.359 cm^2 on day 8, 0.591 cm^2 on day 35), but a rapid progression (3.050 cm^2) on day 90. The right medial iliac LN exhibited a

transient decrease in size (2.401 cm^2 on day 8, 1.840 cm^2 on day 35), but growth had resumed (2.121 cm^2) on day 90.

4.5 Effects of intratumoral hIL-12 EGT on neoangiogenesis

The anti-angiogenic properties of intratumoral hIL-12 EGT were measured via CEUS of the tumor (*Figure 19* and *Figure 20*) and via VEGF-analysis of serum and tumor tissue in all dogs. In total, 9 malignant tumors were evaluated in 9 dogs. With CEUS, sarcomas were less well-vascularized than (adeno)carcinomas. Despite the lower vascularization of sarcomas, a difference in flow pattern could be observed in all tumor types after intratumoral hIL-12 EGT. A significant decrease in tumor blood volume (characterized by the wash-in area under the curve (WiAU)) and blood flow speed (characterized by the wash-in rate (WiR)) was present in all dogs after intratumoral hIL-12 EGT (*Figure 20*). The blood flow speed showed a decreasing trend between days 1 and 8 ($p= 0.071$) and a significant decrease was apparent between days 8 and 35 of treatment ($p= 0.04$) (*Figure 20*). A significant decrease of relative blood volume was present between days 1 and 8 ($p= 0.018$) (*Figure 20*). A remarkable decrease in intratumoral VEGF was present in 2 dogs at day 35, but no clear decreases in VEGF amounts could be detected in the other treated dogs. Nevertheless, the CEUS findings did demonstrate an anti-angiogenic effect in all treated dogs.

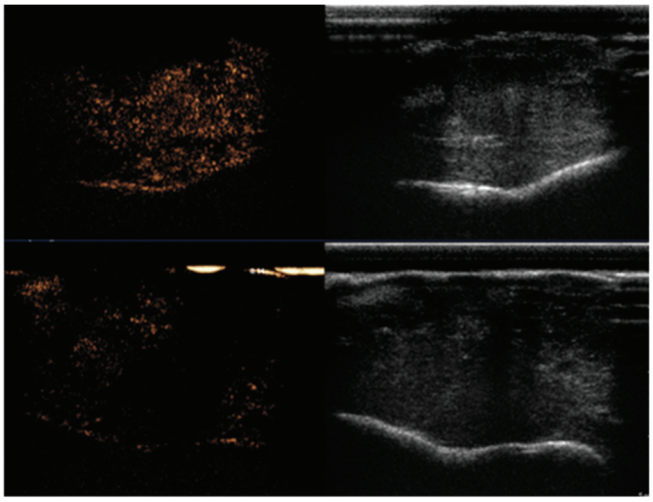


FIGURE 19. Follow-up of tumor vascularization of an interocular mastocytoma (dog 8) on day 1 (top) and 8 (bottom) of intratumoral hIL-12 EGT. The left side of the image corresponds to the CEUS-image of the tumor, the right side to the US B-mode. The decrease in orange speckling (contrast-uptake) is marked enough to be discernable with the naked eye.

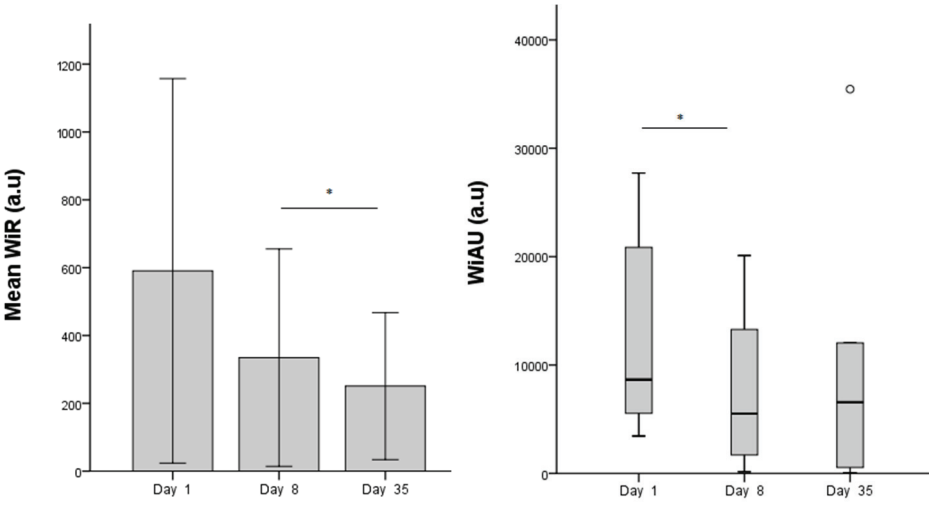


*FIGURE 20. Follow-up of tumor perfusion (WiR) and relative blood volume (WiAU) as measured by CEUS. Mean values ± standard deviations are shown. ° indicates a mild outlier. *, p≤ 0.05 between groups.*

5 Discussion

In this article, we evaluated intratumoral as well as systemic responses to intratumoral hIL-12 EGT in dogs with various tumor types. Immune stimulation was apparent in all treated dogs through either serum IFNγ release or local inflammatory responses within 1 week after hIL-12 pDNA administration. CEUS documented the anti-angiogenic effects of IL-12 therapy in all dogs. In 5 out of 6 dogs, increased hIL-12 levels were measured either in the tumor or serum after intratumoral hIL-12 EGT. In 2 dogs (dog 4 and 7), the tumor biopsy samples did not demonstrate increased hIL-12 levels. For dog 4 this was most likely due to the thin layer of viable tumor tissue present and in dog 7 because the second tumor biopsy was exceptionally taken on day 15 instead of day 8, thereby probably missing the peak of intratumoral hIL-12 production. The peak production of hIL-12 is most likely situated at day 2, as the highest amounts of serum hIL-12 were measured on day 3 after intratumoral hIL-12 EGT.

The first pillar of IL-12 immunotherapy is immune stimulation through activation and recruitment of NK cells, T cells and PMN cells toward the tumor. As local IL-12 pDNA immunotherapy can result in systemic immune activation, changes in pro- and anti-inflammatory cytokines and immune cells were analyzed in the patients' blood samples. Three days after the first intratumoral hIL-12 EGT, a transient decrease in lymphocytes, eosinophils and basophils concurred with a serum increase in IL-12, IFNγ or IL-10. Transient increases of IFNγ (an immunostimulatory cytokine) and IL-10 (an immunosuppressive cytokine) serum levels have been previously described after IL-12 treatment[327, 328] and represent its positive and negative feedback loop, respectively.[329] The balance between the level of IL-12, with IFNγ production and T cell expansion on the one hand, and the level of IL-10, with effector T cell suppression on the other hand, may dictate the end result of the immune response.[329] Interestingly, IL-10 causes significant suppression of IFNγ production in resting peripheral blood mononuclear cells (PBMC), whereas once PBMCs are activated, IL-10 no longer has the ability to suppress their IFNγ production.[330] Therefore, when a pre-existing antitumoral immune

response exists, IL-12 is highly suited to enhance this pre-existing immune response,[331] unhindered by the IL-10 feedback response.

The transient decrease in number of lymphocytes, eosinophils and basophils after the first intratumoral hIL-12 EGT is likely related to their quick recruitment into the tumoral tissue as a result of IL-12- and IFNγ-mediated upregulation of intratumoral vascular adhesion molecules.[213, 327, 332, 333, 334] Indeed, infiltration of CD4+ and CD8+ T cells,[146, 219, 221, 234, 335, 336] NK cells,[334] macrophages[334] and plasma cells[221, 328] has been described following intratumoral IL-12 treatment. Unfortunately, histopathologic confirmation of lymphocytic and/or eosinophilic tumor infiltration has not been performed in our study since this would entail supplementary sedation and trauma to the patients. Nevertheless, the fact that the observed drop in immune cells coincided with the appearance of intratumoral inflammatory symptoms (erythema and swelling) supports the hypothesis that the intratumorally produced hIL-12 recruited immune cells into the tumor. Although electroporation is known to cause swelling, such electroporation-induced swelling subsides within 48 h after electroporation,[226, 337] whereas in our patients the inflammatory symptoms only became apparent after 48 h. An alternative explanation for the transient drop in leukocytes after the first treatment, could be the IFNγ-mediated induction of nitric oxide production from macrophages.[338] Nitric oxide induces suppression of T cell mitogenic responses,[338] possibly clarifying the initial decrease in leukocytes and lag in lymphocyte recovery. Furthermore, the initial decrease in leukocytes could be attributed to an anesthesia-induced stress response,[339] although no further decrease was observed after the first treatment and the dogs were anesthetized for each treatment according to the same protocol.

Although IL-12 initiates a period of immunosuppression, an enhanced protective antitumor immunity develops afterward.[338] Indeed, the initial drop in immune cells was followed by an increase in all treated dogs. Monocytes recovered quickly and briefly demonstrated a peak amount during days 5-8 after intratumoral hIL-12 EGT, whereas recovery above baseline was present at days 15 and 35 for lymphocytes and eosinophils respectively. Since activated T cells[340] and eosinophils[341] express IL-12 receptors, the initial drop in these immune cells followed by their increase above baseline might indicate a continued attraction toward the tumor. Eosinophilia is regularly observed

during immunotherapy protocols[185] and tumor-associated eosinophilia has been linked with a generally good prognostic value.[185] Eosinophils do often exhibit a state of degranulation when in close proximity to tumors.[185] The induced IFNγ may have stimulated enhanced eosinophilic superoxide generation and degranulation.[342] Alternatively, plasmid IL-12 gene therapy has shown to induce a significant amount of cancer cell death[146] and necrosis induces eosinophilic migration.[185] No significant changes for neutrophils were detected, in contrast to a decrease in circulating neutrophils during IL-12 therapy described before.[327] A transient decrease in hematocrit was observed in all dogs throughout the study. The decrease in hematocrit is most likely linked to a slight increase in serum IFNγ, since IFNγ suppresses human erythroid colony-forming units.[343, 344]

The effects of intratumoral hIL-12 EGT on the levels of pro- and anti-inflammatory cytokines in the tumors were also analyzed. Considering IL-12 is a promoter of the Th1 response, the intratumoral Th2 response was likely inhibited, which in turn induced a Th2 serum feedback response. As such, IL-12 probably induced an intratumoral decrease of IL-24, a Th2-cytokine able to selectively kill different cancer cells,[308] while a serum increase of IL-24 was present 3, 8, 15 or 35 days after the first intratumoral hIL-12 EGT and a transient increase of serum IL-10 was present 3 and 8 days after the first intratumoral hIL-12 EGT.

The second pillar of IL-12 antitumoral immunotherapy is anti-angiogenesis, which is induced through the down-regulation of the pro-angiogenic stimulus VEGF[208, 210] and the prevention of development of new vasculature as well as induction of involution of nascent vessels through IP-10 and MIG.[208, 210] The amount of VEGF was measured through ELISA in serum and in tumoral tissue, whereas the effects of IP-10 and MIG were monitored with CEUS. Only 2 dogs demonstrated a remarkable decrease in intratumoral VEGF levels, while the CEUS findings indicated a significant decrease in relative blood volume and blood flow speed in all dogs after treatment. Malignant and benign tumors are characterized by a rapid and slow increase of contrast uptake, respectively.[345] In similar terms, the observed decrease in blood flow speed could

indicate a less aggressive behavior of the tumor after IL-12 treatment. A similar decrease in angiogenesis parameters was also observed after an anti-angiogenic treatment in rats.[346] Although the anti-angiogenic effects of IL-12 can be monitored in various ways (decrease in intratumoral VEGF and microvessel density (MVD),[333, 347] apoptosis of endothelial cells[348]), CEUS provides an excellent non-invasive quantitative and qualitative alternative for biopsies. CEUS has proven to be a more sensitive technique for angiogenesis monitoring than immunohistopathology,[346] as small biopsies are not likely representative for the whole tumor.[227] Moreover, histological follow-up entails invasive and repetitive biopsies within short time spans, which is not required for CEUS.[346]

In this study, hIL-12 was used instead of canine IL-12. Belgian legislation requires all new drugs that are evaluated in clinical trials with canine patients to be of GMP-grade, therefore human IL-12 pDNA was chosen for 2 reasons. First, a GMP grade pDNA encoding canine IL-12 was not available at the time and second, human IL-12 is recognized by canine immune cells[231] without causing a cross-reaction toward canine IL-12.[349] Indeed, canine and human IL-12 share 90% genetic identity based on amino acid sequence analysis[349] and human IL-12 activates proliferation of canine PBMCs in *in vitro* setting and triggers immune responses in canine PBMCs.[231, 232] And, last but not least, intratumoral EGT with a pDNA encoding hIL-12 has yielded antitumoral responses in a subset of treated dogs.[146, 221, 222]

Even though IL-12 transfection was successful and IL-12 was produced by all treated dogs, this did not prohibit further growth of the primary tumors in any of the dogs. However, the consistency of the primary tumors did change after the first or second intratumoral hIL-12 EGT. As ischaemic necrosis often leads to softening of the tumor,[350] the observed softening is likely the result of the anti-angiogenic effects of the IL-12 treatment.

Three owners reported an improvement of quality of life after the third treatment session. Their dog exhibited a more energetic and happier behavior despite progression of the primary tumor. A positive association between immune stimulation and improvement of quality of life has been described in previous immunotherapy trials.[292]

A decrease in well-being is associated with a Th2 profile.[351, 352] Since IL-12 elicits a Th1 response and Th1 and Th2 responses are mutually inhibitory,[352] it is possible that the induced Th1 immune stimulation contributed to the improvement in welfare of several IL-12 treated dogs.

Despite the absence of a clear effect of intratumoral hIL-12 EGT on the primary tumor, we observed in 1 of the 2 patients with LN metastases a transient decrease in size of these metastases. The other patient with LN metastases did not complete the study; therefore the putative anti-metastatic effect of IL-12 could not be evaluated in this dog. A transient decrease in size of LN metastases after intratumoral IL-12 EGT has been previously described by Cutrera et al..[222]

A growth of a primary tumor concurrent with a reduction of its metastases seems paradoxal, but for complete tumor eradication, tumor infiltration with CD8+ T cells is required, whereas prevention of growth of metastases is also regulated by NK cells, B cells and the IFNγ-mediated anti-angiogenesis.[214]

Although remarkable cure rates have been described with intratumoral IL-12 pDNA administration in rodent cancer models,[293, 331, 333] similar successes can thus far not be claimed in dogs and men with spontaneous cancer.[219, 218] In the majority of preclinical studies with mice, the impact that slowly progressing tumors exert on the adaptive immune system is not taken into account.[63] This underscores the importance of evaluating immunotherapeutic treatments in appropriate cancer models such as pet dogs with spontaneous cancer.

So far, the effects of IL-12 therapy in dogs have been evaluated on relatively small tumors of 0.05 to 25.4 cm^3 [146, 221, 222] and intratumoral IL-12 production after IL-12 EGT has proven to be a safe treatment option in humans and pet dogs. [218, 219] However, IL-12 induced cell death could potentially induce tumor lysis syndrome in large tumors (such as those of dogs 1, 3, 4, 5 and 9). It is also possible that, if a large amount of cancer cells produce IL-12, this could lead to high systemic IL-12 values. Grave toxicity has been shown when pure IL-12 was administered systemically to human cancer patients.[213] To this end, possible side effects of the procedure were monitored by regular clinical examination and measurements of selected hematology and biochemistry parameters.

Surprisingly, 2 of the 3 dogs that were treated with IL-12 EGT on day 1, 3 and 5 developed side effects (immune-mediated anemia and thrombocytopenia, dogs 1 and 7 respectively) that could be attributed to IL-12 toxicity. Since Chuang et al. saw a peak level of IL-12 on day 7 following intratumoral electroporation with 1 mg of human IL-12 pDNA in dogs,[146] the precautionary measure was taken to change the treatment regimen and spread the interval between treatments from 1 day to 1 week to avoid IL-12 accumulation in the serum and its possible associated side effects. Although no further adverse effects were observed after the changes in administration schedule, we believe the initial IL-12 EGT regimen was not responsible for the observed adverse effects. Similar or higher serum amounts of IL-12 or IFNγ (the cytokine responsible for IL-12 toxicity) were detected in the dogs receiving IL-12 EGT once a week; yet none of them developed any adverse effects. A more satisfactory explanation takes account of the impact cancer has on the immune system. Cancers can induce auto-immunity through disruption of central tolerance, peripheral immune dysregulation, and alteration of self-antigens. Cancer-induced auto-immune complications (paraneoplastic syndrome) occur in many tumor types. For instance, 10 to 25% of patients with chronic lymphocytic leukemia will develop auto-immune complications, of which auto-immune hemolytic anemia and immune thrombocytopenia are the most common types.[353] It is therefore possible that the observed adverse effects were not a direct consequence of IL-12 toxicity, but represented an aggravation of a pre-existing subclinical paraneoplastic syndrome that was further stimulated by the intratumoral hIL-12 EGT. A 22-fold increase in IL-10 serum levels was measured in 1 dog (dog 3), possibly in an attempt to control the auto-immune response. Any other adverse effects were more likely related to tumor progression than to IL-12 toxicity. It could be interesting to equip pDNA in the future with a regulatory element that enables the control of IL-12 expression in case of doubt.

Although electroporation is a very effective way to acquire gene transfer, the technique has some limitations. First, an adequate electrical conduction, which is crucial for effective perforation of cell membranes, is not always achievable in all tumors. Indeed, some tumors did not demonstrate necrosis on US, but their core exhibited a tissue

resistance below 50 Ohm, inhibiting effective electroporation. In dog 4, for example, only a minimal layer of viable tissue (4-6 mm) could be electroporated. Furthermore, oral tumors are more prone to shearing when injecting the pDNA solution followed by insertion of electrodes. The injected pDNA solution is also prone to leakage in ulcerated tumors. As a consequence, strict application of the calculated injection volume is not always possible, which lowers the treatment efficiency since the extent of gene expression is proportional to the amount of transduced cells and of intact plasmid copies entering the nucleus.[354] Additionally, the induction of an intratumoral electric field is perceived as quite painful and requires adequate analgesia and complete sedation in dogs. Unfortunately, sedation is detrimental to the contemplated immune response as it induces a stress response resulting in immune suppression.[355] Moreover, the anesthesia and adjustment of the intratumoral placement of the electrodes to acquire a feasible electrical tissue resistance (in our hands a minimal of 65 Ohm) are time-consuming. The difficulty in proper electrical conduction and the immunosuppressive and time-consuming anesthesia could be avoided through the use of alternative gene delivery methods such as sonoporation, where US waves are applied to create cell membrane pores. However, this technique requires further technological, biological and medical studies before it can be translated into routine clinical practice.[356]

In our study, a similar haematologic effect of hIL-12 is seen in the 1-day interval and 1-week interval regimen. Numerous explanations are possible, such as IFNγ-induced inhibition of transgene expression of the cytomegaloviral (CMV) promotor of the IL-12 pDNA construct.[357] As IL-12 pDNA induces IFNγ production, this could explain the similar results seen between the dogs treated with a 1-day interval and a 1-week interval. In both groups IFNγ was induced after hIL-12 EGT in the tumoral tissue and in both groups further treatments did not seem to induce an additional decrease in immune cells. This argument is further supported by enhanced transgene expression seen in vivo after administration of neutralizing anti-IFNγ antibodies.[338, 357] Furthermore, a biopsy taken on day 15 from dog 7 did not show the presence of IL-12, whereas a clear increase in serum IL-12 was measured on day 3. An attenuation

response due to formation of antibodies against hIL-12 is also feasible since Paoloni et al. found in 8 out of 12 canine melanoma patients production of antibodies against human IL-12 2 to 4 weeks after administration of NHS-IL-12, an immunocytokine consisting of an antibody linked to human IL-12. The host antibody responses were directed mainly against the human IgG part of the molecule, but 5 out of 14 dogs tested also developed antibodies against the human IL-12 portion.[227] Other possible causes include a dilution effect due to the loss of non-integrated DNA after cancer cell multiplication or destruction by endonucleases present in the cancer cells,[357] but then a similar haematologic effect would be expected after a second treatment.

6 Conclusion

In conclusion, tumor biology is extremely complex and various factors influence the process of carcinogenesis and progression. Although the immune system is capable of mounting an immune response against cancer cells, this response is often not robust enough to eliminate them. To enhance the efficacy of the immune response toward the tumor, immunostimulatory cytokines can be administered.[331] Interleukin 12 is a cytokine that enhances a pre-existing antitumor immune response through stimulation of NK and T cells[331] and also targets various aspects of tumor angiogenesis through induction of IFNγ and its cascade effects.[208] In this article, immune stimulation was apparent in all treated dogs through local inflammatory responses within 1 week after the first intratumoral hIL-12 EGT and anti-angiogenic effects were documented through CEUS in all treated dogs. Although intratumoral IL-12 EGT has shown to be able to cure human patients without having a negative impact on the host (in contrast to cytotoxic treatments such as chemotherapy),[219] IL-12 monotherapy of spontaneous (large) tumors has not yielded clinically relevant results in the pet dogs of this study. As a monotherapy, this therapy offers cytostatic rather than cytotoxic results. Interleukin 12 is especially useful as adjuvant therapy in metastatic cancer or prevention of metastases. For eradication of established tumors through IL-12, the presence of a pre-existing antitumoral T cell response is required. In cancer-bearing patients, only a limited number dispose of a pre-existing immunity.[331] This provides a rationale to

believe that the effectiveness of IL-12 therapy could be enhanced by vaccinating the patient against his own cancer cells prior to IL-12 therapy.[331] As an alternative, the efficacy of IL-12 therapy could be enhanced by combining it with metronomic cyclophosphamide to selectively decrease the amount of Tregs,[285] since IL-12 effects are transient and induce a rebound in Tregs.[358] Furthermore, immunotherapy generally has a more powerful effect in a minimal residual disease setting,[147] thus IL-12 therapy could benefit from a combinatorial strategy with surgery.

7 Supplements

7.1 Supplementary table 1

Intratumoral and serum absorbance (OD) values of IL-12, IFNγ, IL-10, IL-24 and VEGF prior, during and after intratumoral IL-12 pDNA treatment.

Intratumoral IL-12 absorbance			
	Day 1	Day 8	Day 35
Dog 1*	*0.109*	0.352	*0.106*
Dog 2*	*0.112*	1.087	*0.153*
Dog 4	*0.126*	*0.106*	*0.090*
Dog 5	*0.108*	0.209	*0.111*
Dog 6	*0.109*	0.349	*0.096*
Dog 7°	0.087	0.074	0.082

Serum IL-12 absorbance					
	Day 1	Day 3	Day 8	Day 15	Day 35
Dog 1*	*0.129*	0.371	*0.212*	*0.091*	*0.089*
Dog 2*	*0.128*	*0.161*	*0.127*	*0.122*	*0.128*
Dog 4	*0.110*	*0.134*	*0.114*	*0.108*	*0.105*
Dog 5	*0.108*	*0.113*	*0.118*	*0.125*	*0.111*
Dog 6	*0.117*	*0.169*	*0.146*	*0.149*	*0.125*
Dog 7°	0.080	0.934	0.236	0.106	0.121

Intratumoral IFNγ absorbance			
	Day 1	Day 8	Day 35
Dog 1*	*0.072*	0.101	0.083
Dog 2*	*0.066*	0.279	*0.065*
Dog 4	*0.065*	0.077	*0.071*
Dog 5	*0.063*	0.077	*0.063*
Dog 6	*0.070*	0.083	0.080
Dog 7°	0.053	0.042	0.049

Serum IFNγ absorbance					
	Day 1	Day 3	Day 8	Day 15	Day 35
Dog 1*	*0.039*	0.077	*0.038*	*0.030*	*0.031*
Dog 2*	*0.039*	*0.048*	*0.040*	*0.042*	*0.041*
Dog 4	*0.034*	*0.043*	*0.049*	*0.029*	*0.033*
Dog 5	*0.048*	*0.039*	*0.039*	*0.038*	*0.035*
Dog 6	*0.031*	*0.035*	*0.040*	*0.063*	*0.041*
Dog 7°	*0.017*	*0.055*	*0.015*	*0.015*	*0.016*

Intratumoral IL-10 absorbance			
	Day 1	Day 8	Day 35
Dog 1*	0.022	0.016	0.023
Dog 2*	0.016	0.016	*0.017*
Dog 4	0.018	0.010	0.011
Dog 5	0.013	0.013	*0.009*
Dog 6	0.036	0.028	0.037
Dog 7°	0.033	0.023	0.028

Serum IL-10 absorbance					
	Day 1	Day 3	Day 8	Day 15	Day 35
Dog 1*	*0.041*	0.054	0.096	*0.039*	*0.038*
Dog 2*	*0.012*	0.086	0.078	*0.026*	*0.021*
Dog 4	*0.017*	*0.016*	*0.036*	*0.012*	*0.038*
Dog 5	*0.044*	0.056	*0.022*	*0.016*	*0.015*
Dog 6	*0.044*	0.056	*0.040*	*0.036*	*0.040*
Dog 7°	*0.010*	0.076	*0.012*	0.025	*0.010*

Intratumoral IL-24 absorbance			
	Day 1	Day 8	Day 35
Dog 1*	1.185	0.867	0.862
Dog 2*	0.932	0.937	0.924
Dog 4	0.944	1.101	0.981
Dog 5	0.939	0.911	0.877
Dog 6	1.228	1.072	0.958

Serum IL-24 absorbance					
	Day 1	Day 3	Day 8	Day 15	Day 35
Dog 1*	0.997	1.184	1.141	0.783	1.163
Dog 2*	0.891	0.943	1.194	0.974	0.971
Dog 4	1.238	1.107	1.080	1.365	1.607
Dog 5	1.037	1.030	1.022	1.042	1.121
Dog 6	1.001	1.122	1.144	1.034	1.016

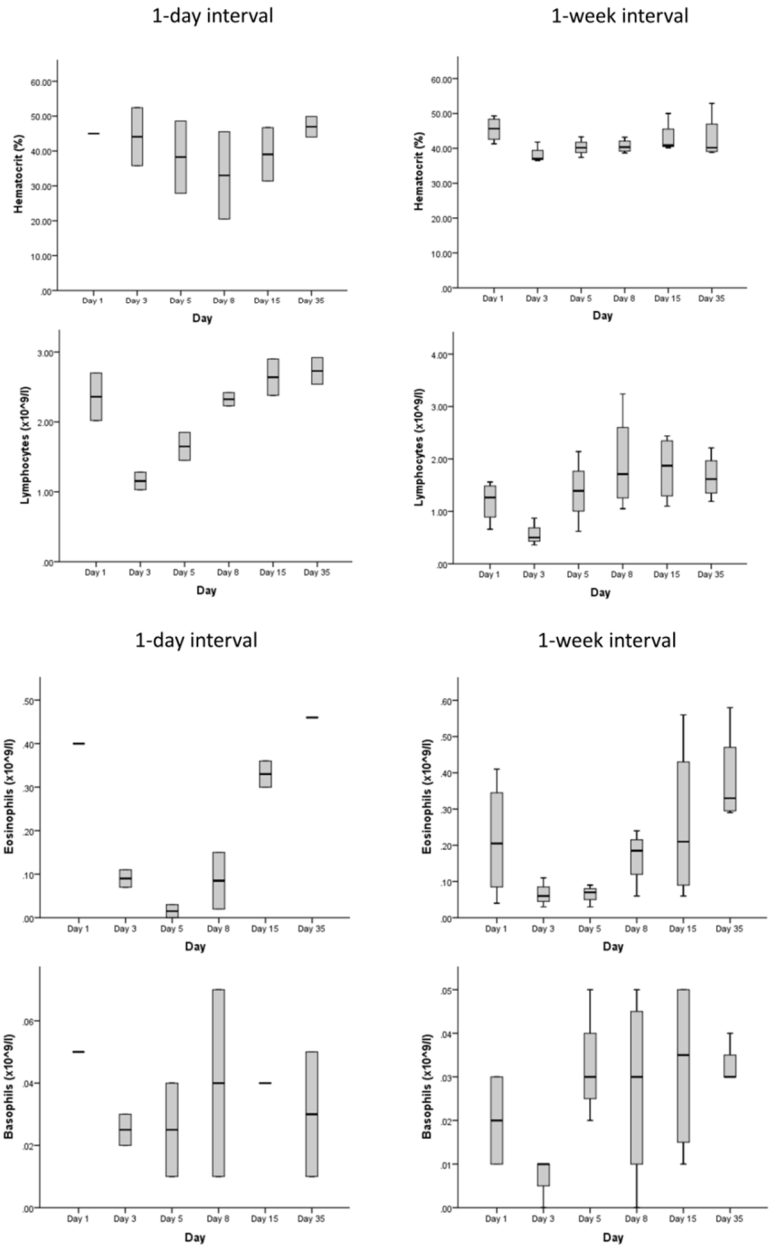
Intratumoral VEGF absorbance			
	Day 1	Day 8	Day 35
Dog 1*	*0.044*	*0.054*	*0.043*
Dog 2*	*0.051*	0.080	*0.060*
Dog 4	0.524	0.535	0.217
Dog 5	*0.060*	*0.053*	0.210
Dog 6	0.068	*0.042*	*0.049*
Dog 7°	0.140	0.104	0.044

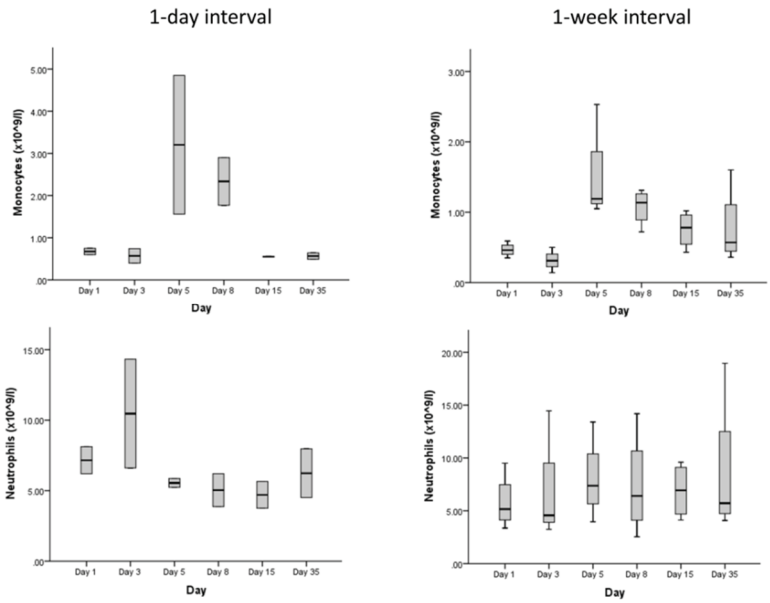
Serum VEGF absorbance					
	Day 1	Day 3	Day 8	Day 15	Day 35
Dog 1*	*0.033*	*0.042*	*0.032*	*0.031*	*0.040*
Dog 2*	*0.039*	*0.057*	*0.022*	*0.019*	*0.020*
Dog 4	*0.030*	*0.034*	*0.034*	*0.032*	*0.026*
Dog 5	*0.023*	*0.016*	*0.025*	*0.022*	*0.022*
Dog 6	*0.024*	*0.026*	*0.028*	*0.027*	*0.030*
Dog 7°	0.018	0.025	0.018	0.016	0.018

*Values in italic represent values below the standard curve, underlined values within the standard curve, *: 1-day regimen; °: dog 7 was analyzed on a different ELISA plate. A change in OD with ≥ 0.05 was considered as a decrease or increase of that cytokine.*

7.2 Supplementary figure 1

Complete blood count in dogs that completed a whole 1-day or 1-week interval regimen





Complete blood count in dogs that completed a whole 1-day interval regimen (n= 2) and 1-week interval regimen (n= 4). Temporary decrease in hematocrit and amount of peripheral lymphocytes (day 1-3), eosinophils (day 1-5) and basophils (day 1-3) following intratumoral hIL-12 EGT. Temporary increase in amount of peripheral monocytes (day 3-15) and neutrophils (day 1-5) following intratumoral hIL-12 EGT.

7.3 Supplementary data 1: Response to treatment

Despite hIL-12 EGT, all primary tumors continued to grow. A decrease in tissue resistance (Ohm) as measured by the electroporator was observed in 7 out of 8 dogs (all but dog 3). Due to extreme tumor heterogeneity, the tissue resistance in dog 7 could not be evaluated. Further tumor specifics are discussed per patient that received a whole treatment cycle.

Dog 1: The soft tissue sarcoma was firm before treatment, but softened by day 8. Erythema and swelling were present 2 days after the first intratumoral hIL-12 EGT (*Figure 18*). The consistency of the tumor remained soft and elastic thereafter. Prior to

treatment, the owners reported the dog was excessively licking the tumor. The dog discontinued this behavior 20 days after his third intratumoral hIL-12 EGT to date (16 months of follow-up to date). The owners reported that the dog showed an ameliorated appetite, and increased weight and a general increase in well-being.

Dog 2: The intra-oral fibrosarcoma did not demonstrate a discernable change in consistency after IL-12 treatment. The mass remained stable in size for 1 month after treatment, after which growth resumed. The dog was in generally good shape and no clinically relevant changes occurred for 8 months. After 9 months, occasional nose bleedings became apparent and the dog was euthanized.

Dog 4: The very aggressive mammary carcinoma had a promising response to the IL-12 treatment, since, 2-3 days after each treatment, impressive signs of local inflammation were present (*Figure 21*). By day 8, all nodules had become softer. At day 9, the skin between tumor nodules sheared and released tumor exsudate. The tumor exsudate was clear and slightly bloody, and the owners reported a necrotic odor. The dog developed a fever of 39.8°C at day 10, which spontaneously subsided within 24 hours. From day 12 on, the openings between the nodules released great amounts of odorless exsudate. The openings narrowed by day 20. At day 32, the exsudate was chunky and the necrotic odor reappeared. The dog's well-being regressed from that point on, which led to the decision of euthanasia.

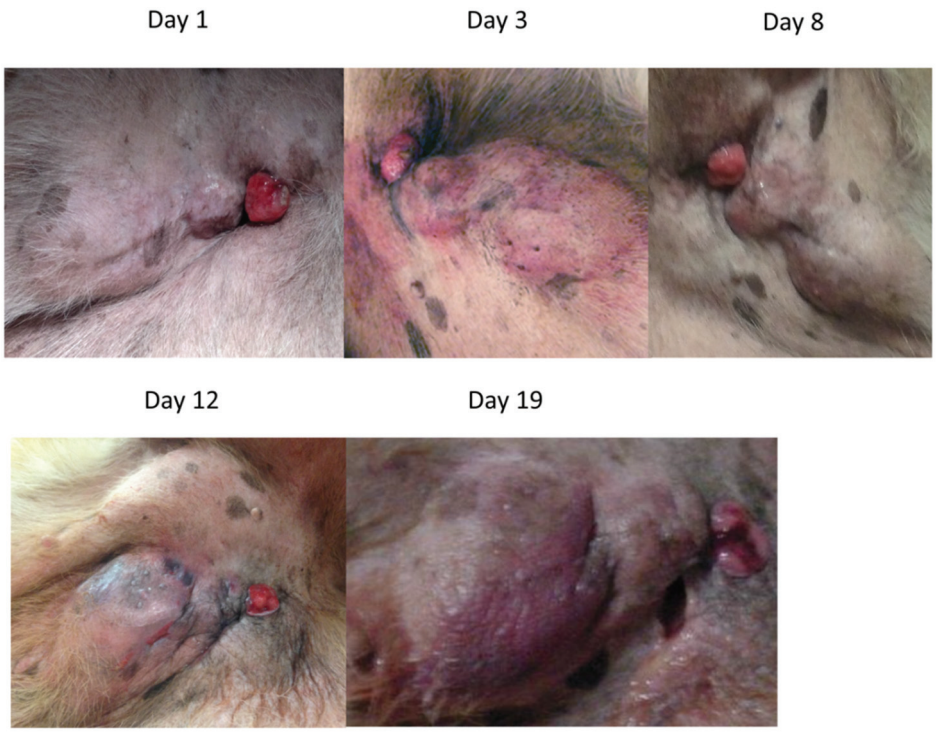


FIGURE 21. Tumor inflammation after intratumoral hIL-12 EGT in a tubulopapillar complex carcinoma of the mammary glands (dog 4). The tumor inflammation is recognized by erythema on day 3 and swelling on day 8 and 12. Day 1: before treatment, day 3, 8, 12, 19: after treatment initiation (1-week interval regimen).

Dog 5: The intra-oral fibrosarcoma did not demonstrate a discernable change in consistency after IL-12 treatment. The tumor was associated with bone destruction of the right maxilla, palatine and sphenopalatine bone, and invasion of the nasopharynx. During treatment, the dog demonstrated loss of appetite, most likely due to tumor progression. The dog was euthanized on day 52 due to progressive weakness.

Dog 6: The perianal adenocarcinoma easily bled on day 1, 8 and 15 after rectal toucher. At day 15, the tumor was erythematous and swollen (*Figure 22*). From day 15 on, the dog gained weight and experienced fewer difficulties defecating (less straining). On day 35, no bleeding occurred after rectal toucher. The left medial iliac LN demonstrated

slow tumor growth between days 8 and 35, but quintupled in size by day 90. The right iliac LN demonstrated partial regression (more than 20% decrease in tumor size) between days 8 and 35, but tumor growth resumed by day 90. The dog was euthanized on day 210.

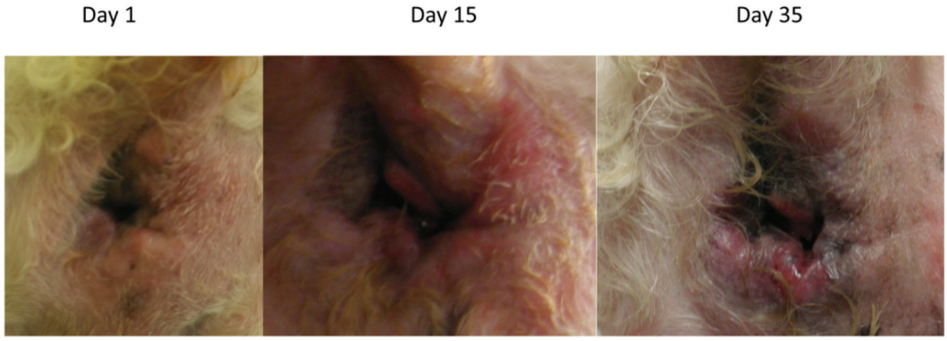


FIGURE 22. Tumor inflammation after intratumoral hIL-12 EGT in a perianal adenocarcinoma (dog 6). The tumor inflammation is recognized by erythema and swelling on day 15. Day 1: before treatment, day 15, 35: after treatment initiation (1-week interval regimen).

Dog 7: The schwannoma was firm before treatment, but softened by day 15, although less spectacularly than in dog 1. Erythema was present on day 3 and swelling during days 5-8 (*Figure 23*). The dog remained in generally good shape and no further changes occurred to date (11 months).

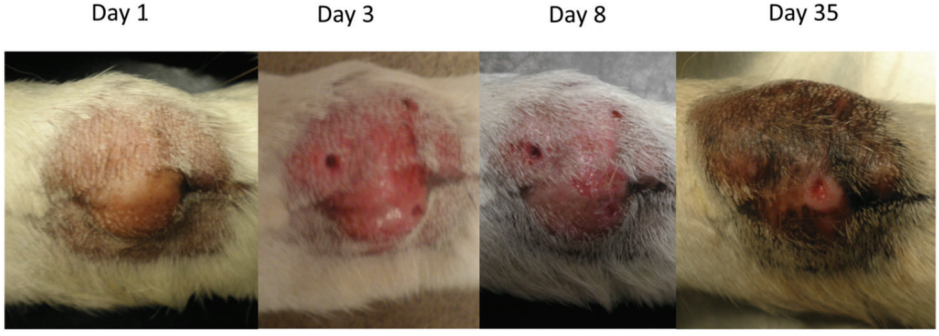


FIGURE 23. Tumor inflammation after intratumoral hIL-12 EGT in a schwannoma dorsal to the carpus (dog 7). The tumor inflammation is recognized by erythema and swelling on day 3. Day 1: before treatment, day 3, 8, 35: after treatment initiation (1-week interval regimen).

CHAPTER 3

Interleukin 12 and metronomic chemotherapy in cancer-bearing pet dogs

1 Abstract

The immunological, anti-angiogenic and clinical effects of metronomic cyclophosphamide and 3 consecutive intratumoral IL-12 gene therapy (EGT) treatments were evaluated in 6 dogs with spontaneous cancer. A total of 3 tumor biopsies (day 1, 15, 35) and 5 blood samples (day 1, 3, 8, 15, 35) per dog were taken prior, during and after intratumoral IL-12 EGT. In all dogs, an initial decrease in blood immune cells was followed by an increase 1-3 weeks after treatment initiation. The decrease in peripheral leukocytes 2 days after IL-12 EGT coincided with erythema and swelling of the tumor. In the tumor, a transient increase in IL-12 levels was measured, whereas a continuous increase in interferon γ (IFNγ) and thrombospondin 1 (TSP-1) were determined in contrast to a continuous decrease in vascular endothelial growth factor (VEGF). In the serum, a transient increase in IL-12 and interleukin 10 (IL-10) levels were noted in contrast to a transient decrease in VEGF and TSP-1. There were no changes in intratumoral IL-10 levels or serum IFNγ levels. Microbubble contrast-enhanced ultrasound (CEUS) was performed prior, during and 3 weeks after the last intratumoral IL-12 EGT treatment (on day 1, 8 and 35) to establish the anti-angiogenic effect of IL-12 EGT. The treatment resulted in a significant decrease of tumor relative blood volume and blood flow speed. All primary tumors continued to progress in time, but the CEUS indicated that this progression was slower than before treatment. Besides the encouraging immunostimulatory and anti-angiogenic effects observed in all dogs we also noticed in 4 out of 6 dogs clinically relevant improvements in quality-of-life and weight. However, tumor regression could not be obtained. The laboratory and ultrasound (US) results hold great promise for combinatorial strategies of IL-12 EGT and metronomic chemotherapy with conventional antitumor (immuno)therapies.

2 Introduction

The development of cancer is recognized by the body's immune system as an anomaly. On a genetic level, many control mechanisms are in place to detect and destroy aberrant cell copies. Similarly, immune surveillance plays an important role in the

prevention of neoplastic spread.[38] Unfortunately, despite these checkpoints for abnormal cell development, cancer cells are able to escape their control resulting in the development of a tumor.[42] The relationship between cancer and the oncologic patient's immune system is very complex and continuously evolving.[359] It is therefore unlikely that one immunotherapeutic agent will be powerful enough to produce an effective antitumoral immune response. The more cancer promoting pathways that are simultaneously targeted, the higher the chances of successfully decreasing tumor escape.[360] We propose to combine the antitumoral effects of interleukin 12 (IL-12) with metronomic cyclophosphamide (CP) as a multi-targeted and complementary approach. IL-12 is a powerful immunostimulatory cytokine that links innate and adaptive immune responses. By stimulating the immune response, IL-12 is able to activate key immune cells such as natural killer (NK) and T cells to recognize and kill cancer cells.[197] Furthermore, IL-12 decreases the suppressive function of myeloid-derived suppressor cells (MDSCs)[207] and inhibits the differentiation of T cells to regulatory T cells (Tregs) as well as the expansion of Tregs.[205, 206]

Metronomic chemotherapy, i.e. the continuous administration of low-dose chemotherapeutics, has much potential in combinatorial treatments[263] and possibly synergizes with other immunomodulatory or anti-angiogenic antitumoral therapies.[286] The aim of metronomic chemotherapy is not to kill cancer cells directly but indirectly, by influencing the microenvironment of the cancer cells. Cyclophosphamide is a well-known alkylating chemotherapeutic that has been mainly used in a cytotoxic dose to kill rapidly multiplying cells such as cancer cells.[263] When given in metronomic doses, CP is able to selectively decrease the amount of circulating Tregs and has anti-angiogenic properties.[266]

There are multiple advantages to the combination of intratumoral IL-12 EGT and metronomic CP as both therapies decrease immunosuppression and angiogenesis. Whereas metronomic CP has shown to selectively decrease the amount of Tregs, [293, 279, 361] IL-12 decreases the suppressive function of immunosuppressive MDSCs,[207] which could compensate for the CP-induced MDSC expansion.[361] The anti-immunosuppressive effects of metronomic CP restores NK cell effector functions and

thus facilitates the generation of innate and eventually adaptive immune responses induced by IL-12.[362] Indeed, the combination of a low dose of CP and IL-12 induced a stronger Th1 response than IL-12 alone in mice.[331] Furthermore, multiple complementary angiogenesis pathways are simultaneously targeted through this combinatory approach. Interleukine 12 inhibits neo-angiogenesis via induction of interferon γ (IFNγ) and its cascade products Inducible Protein (IP) 10 and Monokine Induced by Gamma interferon (MIG).[208] On the other hand, metronomic CP causes apoptosis of capillary endothelial cells by up-regulating the endogenous angiogenesis inhibitor thrombospondin 1 (TSP-1).[272] In turn, TSP-1 binds not only to the CD36R on endothelial cells inducing apoptosis,[273] but also to the CD47-part of the VEGFR, thus blocking the proangiogenic effects of VEGF on endothelial cells.[274]

Because of severe toxicity, systemic injection of recombinant IL-12 (rIL-12) is not recommended.[213] Local (intratumoral) injection of rIL-12 has been considered, but requires frequent injections due to the short half-life of rIL-12 (12 h).[363] Therefore, intratumoral IL-12 gene therapy, using plasmid (p)DNA electroporation, has been evaluated as it offers a prolonged delivery of IL-12 and resulted in very promising (pre)clinical results.[219, 220, 221, 222] Electroporation is the short administration of electrical pulses to ensure the formation of transient pores into the plasma membrane of cells thereby facilitating the intracellular delivery of large molecules such as pDNA.[215] Once the pDNA reaches the cell's nucleus, the production of the protein encoded on the plasmid ensues.[216, 217] *In vivo* electroporation does not cause any severe adverse events,[218] and several canine and human clinical trials showed the safety and clinical efficacy of electroporation-mediated intratumoral delivery of a pDNA encoding IL-12.[219, 220, 221, 222]

In this article, the effects of intratumoral IL-12 EGT in combination with metronomic CP on tumor angiogenesis, amount of circulating Tregs and shifts in the tumor microenvironment were studied in 6 dogs with various tumor types and sizes. To our knowledge, this is the first article that describes the combinatorial effects of

intratumoral IL-12 EGT and metronomic CP (IL-12-CP treatment) in dogs with spontaneous tumors.

3 Materials and Methods

3.1 Animal selection

Client-owned dogs with spontaneous malignant neoplasms were enrolled between April 2015 and October 2015. Patients included in the study were not eligible for surgery, either due to recurrent disease for which conventional surgery was already exhausted, or due to the impractical location of the tumor. Other conventional therapies such as chemotherapy or radiation were not given prior to IL-12-CP treatment.

The study cohort consisted of 6 dogs, 2 intact and 2 castrated males and 2 spayed females belonging to 5 different breeds (Golden Retrievers (n=2), Stabyhoun, Malinois, French Bulldog and Beagle), their age ranging from 7 to 14.8 years (mean 10.2 years ±2.6) (*Table 12*). The following additional inclusion criteria were applied: a histologically confirmed neoplasia accessible for application of electrodes, normal cardiovascular function, good general health status and a biochemistry profile within reference limits. In all animals, screening for metastatic disease was accomplished by thoracic radiographs and/or abdominal US.

Animals were free of non-steroidal anti-inflammatory drugs (NSAID) at least 1 week before and during the entire study period, except dog 3 who received NSAIDs during the first 3 days of IL-12-CP treatment, since this dog underwent surgical excision of the primary tumor and metastases 1 day before onset of the IL-12-CP treatment. All animal handling and treatment procedures were approved by the Ethical Committee (approval number 2014/81) of the Faculty of Veterinary Medicine of Ghent University, Belgium and the Deontological Committee of the Federal Public Service of Health, Food Chain Safety and Environment, Belgium. A written informed consent was obtained from each dog owner.

TABLE 12. Signalment, tumor type, tumor size and metastasis status of the dogs treated by intratumoral IL-12 gene therapy and metronomic cyclophosphamide

Dog	Breed	Sex	Age	Tumor type (localization)	Tumor size	Metastases
1	Golden Retriever	Fn	14y	Amelanocytic melanoma (intra-oral)	50.24 cm^3	Lymph node cytology and liver ultrasound positive
2	Golden Retriever	Mn	14y 10m	Schwannoma (dorsal to carpus)	360 cm^3	Lymph node cytology and thoracic free
3	Stabyhoun*	Fn	7y	Adenocarcinoma (anal sac)	NA	Abdominal metastasized lymph nodes resected, hypogastric lymph node cytology positive, thoracic free
4	Malinois°	M	11y	Fibrosarcoma (dorsal to carpus)	43.96 cm^3	Lymph node cytology and thoracic free
5	French Bulldog	Mn	8y 11m	Osteosarcoma (costal)	201 cm^3	No, locally invasive in thorax
6	Beagle	M	10y 2m	Fibrosarcoma ((rostro)mandibular)	14.13 cm^3	Lymph node cytology and thoracic free

***F:** female; **Fn:** female neutered; **M:** male; **Mn:** male neutered.*

** no contrast-enhanced ultrasound was performed in this dog*

° this dog received a second IL-12-CP treatment 5 months after the first IL-12-CP treatment cycle.

3.2 Plasmid

An IL-12 pDNA encoding human IL-12 (hIL-12) was obtained via Celsion-EGEN, Inc. (Huntsville, AL, USA). It was produced under GMP conditions by Eurogentec (Seraing, Belgium). The hIL-12 plasmid contains an immediate early enhancer and promoter derived from cytomegalovirus (CMV), 5' untranslated region (UTR), synthetic intron, human p35 gene, human growth hormone (hGH) 3' UTR and polyadenylation signal sequence, CMV promoter, 5' UTR, synthetic intron, human p40 gene, hGH 3' UTR and polyadenylation signal sequence. Of note is the fact that both the hIL-12 subunits are

individually under the control of 2 separate CMV promoters. The sequences of the p35 and p40 cDNAs are identical to the sequences found in GenBank with the exception of a single base mutation in the second codon of the p35 cDNA. The change was made to insert a KOZAK sequence for proper translation initiation and also created an Nco I restriction digest site. The mutation changed the second amino acid of the p35 signal sequence from a cytosine (CYS) to a glycine (GLY). A similar change was made to the second codon of the p40 cDNA.

3.3 Metronomic cyclophosphamide

Dogs received 12.5 mg/m² CP (Endoxan, Baxter, Belgium, Lessines) daily. The dosage of CP was based on a previous study with metronomic CP in dogs performed by our lab.[285] Reformulation of the drugs was done in collaboration with the Faculty of Pharmaceutical Sciences of Ghent University.

3.4 Ultrasound and Contrast-Enhanced Ultrasound

Prior to lL-12 EGT, B-mode US of all tumors was performed with a linear 12-5 MHz or 17-5 MHz transducer (Philips, iU22 xMATRIX, Philips Medical systems, Bothell, Washington, USA) to determine the echogenicity, to assess the presence of mineralization within the tumor and to measure the tumor dimensions.

Following conventional US examination, tumor perfusion measurements with CEUS were performed with the same dedicated machine (iU22 xMATRIX) equipped with contrast-specific imaging technology using a linear 12-5 MHz probe. To evaluate the tumor perfusion, dogs were imaged with manual restraint or, in one dog, under mild sedation with butorphanol (Dolorex® 0.2 mg/kg), a drug that is known not to influence the CEUS.[324] Prior to injection of microbubbles (MB), several imaging parameters were adjusted to decrease early destruction of the injected MBs by the US beam. Mechanical index was decreased (0.09), persistence was switched off and a single focal spot was placed under the tumor.

The MB contrast agent (Sonovue®, Bracco, Milan, Italy), consisting of lipid-stabilized

MBs with a sulfur hexafluoride gas core, was injected intravenously (IV) as a bolus (0.04 mL/kg), immediately followed by a 1 mL flush of sterile saline (Mini-Plasco NaCl 0.9%, Braun) via a peripheral venous catheter inserted in the cephalic vein. Between the boluses, the remaining MBs were destructed by increasing the acoustic power in the imaging plane for several minutes.

During CEUS, areas that should be avoided during injection of the IL-12 pDNA solution, such as main tumor vessels or necrotic tissue, were identified. All CEUS studies were digitally registered as a movie clip at a rate of 10 frames/sec for 90 sec after bolus injection. These clips were analyzed using integrated dedicated specialized computer software (VUEBOX, Bracco) for objective quantitative analysis. Tumor regions-of-interest (ROIs) were manually drawn and mean pixel intensities and time-intensity curves created per ROI. These curves were analyzed for Radius Basis Function parameters representing blood volume (area-under-curve) and blood velocity (sec). A B-mode image was projected simultaneously with CEUS imaging, and (CE)US was repeated for comparison at the identical tumor location at day 8 and 35. CEUS imaging was performed in all dogs, except dog 3, since the tumor and its metastases were surgically removed before IL-12-CP treatment.

3.5 Interleukin 12 electrogene therapy

One mg of IL-12 plasmid per session was diluted with calcium and magnesium free phosphate-buffered saline (DPBS) (14040-174, Invitrogen). The injection volume was 1/6 of the tumor volume that was calculated with the following formula: L*H*Wπ/6 (L: length, H: height, W: width). The sterile IL-12 pDNA injection volume was administered intratumorally throughout the whole tumor mass. An 8-needle array electrode connected to an electroporator (Agile Pulse generator, BTX® Harvard Apparatus) was inserted within 2 min after injection around the injection site. Two pulses of 450 V/cm were given (pulse duration was 0.05 msec and the interval between the pulses 0.2 msec). After 50 msec, those 2 pulses were followed by 8 pulses of 100 V/cm (pulse duration was 10 msec and the interval between pulses 20 msec). The tumor volume treated was restricted by the needle length of the electrodes. Since the

maximum length of the electrodes was 25 mm, tumor tissue at a depth greater than 25 mm could not be electroporated.

3.6 Clinial trial design

Prior to each treatment session, subject owners were interviewed and each dog was physically examined. Blood was collected to assess the subject's hematology and biochemistry. Baseline bloodwork consisted of a complete blood count (CBC) with differential white blood cell count, which was performed using an automated laser hematology analyzer (Procyte, Idexx, The Netherlands) with species-specific software (Vetlab Station). The automated chemistry analyzer Technicon RA-XT (Catalyst, Idexx, The Netherlands) was used for the determination of the following biochemical parameters: blood urea nitrogen, creatinine, serum alkaline phosphatase (SAP) and alanine aminotransferase, glucose, total protein, and globulines. When all values were within reference range, a catheter was placed. Hair overlying tumor nodules was clipped prior to (CE)US. After a general US examination of the region of the tumor, patients received IV contrast agent for CEUS evaluation of tumor perfusion. Afterward, the patient was anesthetized for the electroporation of the IL-12 pDNA. For this purpose, patients were premedicated with buprenorphine (Vetergesic® 10 µg/kg) and after 15 min dexmedetomidine (Dexdomitor® 5 µg/kg) was administered. After another 15 min, anesthesia was induced with propofol (Propovet multidose® 1 mg/kg) and was, if necessary, maintained with isoflurane.

Immediately after IL-12 EGT, 3 cylindrical punch biopsies of 2x8 mm (Bap Medical, 33-31) were taken from different locations within the neoplasm to factor in intratumoral heterogeneity.[325] The biopsies were taken after electroporation to avoid leakage of the injected pDNA from the tumor. After the biopsy taking, the subjects were antidoted with atipamezole (Revertor®, 25 µg/kg). No (N)SAIDs were administered, to prevent interference with the development of an inflammatory response to the treatment. Other non-anti-inflammatory pain medications such as Tramadol (Tramadol EG®, 2 mg/kg) were offered as alternative.

Oral metronomic CP (12.5 mg/m², Endoxan, Baxter, Belgium, Lessines) was initiated at day 1 and maintained at least until day 35.

To assess possible side effects of the procedure, physical examinations of patients were performed at least on day 1, 3, 8, 15 and 35 of the treatment cycle. During examination, the tumor was evaluated for signs of local inflammation (erythema, swelling, pain), secretions, necrosis, etc. The production of hIL-12 and the occurrence of systemic effects were evaluated by measuring the amount of canine IFNγ, IL-10, VEGF and TSP-1 in serum and by screening for aberrations in CBC and/or the biochemistry profile. The response to therapy was assessed by means of repeated measurements of tumor size and perfusion through (CE)US and repeated measurements of the amount of IFNγ, IL-10, VEGF and TSP-1 in tumor tissue (*Figure 24*).

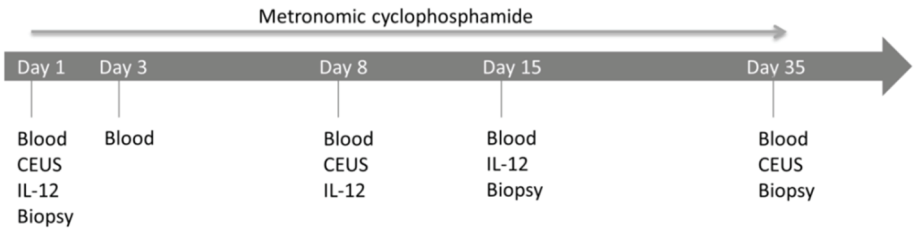


FIGURE 24. Schedule of IL-12-CP treatment. Blood: complete blood count and biochemistry analysis, CEUS: contrast-enhanced ultrasound, IL-12: intratumoral injection of 1 mg hIL-12 plasmid DNA and electroporation, Biopsy: collection of 3 small tumor biopsies.

3.7 Tumor tissue analysis

The biopsies were immediately kept on dry ice and stored in a -150°C freezer (Panasonic, TCPS) until further use. A day prior to ELISA analysis biopsies were thawed in a 600 µl mixture of DPBS and protease-inhibitor cocktail (cOmplex Mini, Roche, 5892970001). Biopsy samples were disrupted with a tissue disruptor (TissueRuptor®, Qiagen) during 30 sec to lyse the cells. The lysed cells were centrifuged at 12,000xg during 10 min at 4°C. Afterward, the supernatant was removed and stored at 4°C until use the following day. The concentration of the cytokines is shown as pg

(IL-12, IFNγ, IL-10, VEGF) or ng (TSP-1) per cm^3 of tumor tissue (*Figure 25* and *Figure 33*).

3.8 ELISA of local and systemic cytokines during IL-12-CP treatment

The presence of canine IFNγ, VEGF, IL-10 and TSP-1 in serum and tumor tissue was determined using ELISA kits (Canine IFN-γ/VEGF/IL-10 Quantikine ELISA Kit, R&D Systems, CAIF00/CAVE00/CA1000, USA and TSP-1 ELISA Kit, Biotang, CA0214, USA respectively) and hIL-12 with Human IL-12 (p70) ELISA MAX™ Standard (Biolegend, 431701, USA). In order to assess if the IL-12 pDNA transfection was successful, the amount of hIL-12 in the tumor was measured for each dog on day 1, 15 and 35 of the treatment. The stimulatory effects of hIL-12 were determined by analysis of the amount of canine IFNγ, while the anti-angiogenic effects were determined by analysis of the pro-angiogenic growth factor VEGF and the anti-angiogenic glycoprotein TSP-1. The anti-immunosuppressive effects were determined by analysis of IL-10. Intratumoral cytokines were assessed on day 1, 15 and 35, while the serum cytokine levels were measured on day 1, 3, 8, 15 and 35 for each dog.

3.9 Determination of percentage of regulatory T cells

Blood was collected before treatment, after 2 weeks and 5 weeks of IL-12-CP treatment in an EDTA tube. The same day, peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll density gradient (GE healthcare Europe, 17-1440-02). The isolated PBMCs were dissolved at 10^7 cells/mL in 40% phosphate buffered saline, 50% fetal calf serum and 10% dimethylsulfoxide (Invitrogen, D12345). Vials were placed in a cryobox at -80°C overnight and then transferred to -154°C. Upon analysis, vials were thawed in a warm water bath and warm medium was gradually added. After 2 wash steps cells were stained for Treg. One million cells in a volume of 100 µL of FACS staining buffer (BD Biosciences, 554656) were used for immunostaining. Cells were stained for the surface antigens CD4 and CD25, using FITC-conjugated anti-dog CD4 (eBioscience, 11-5040-42) and eFluor 660-conjugated anti-dog CD25 antibodies

(eBioscience, 50-0250-42). Surface staining was done at 4°C for 30 min. Intracellular staining for FoxP3 was done as described previously,[285] with a cross-reactive murine PE-conjugated FoxP3 antibody and the permeabilization and fixation/permeabilization buffer that accompanies the antibody (foxP3 staining set, clone fjk-16s, eBioscience). Briefly, after washing to remove unbound surface antibodies, cells were resuspended in fix/perm buffer and incubated at 4°C overnight. The next day, cells were washed twice with permeabilization buffer and incubated with the FoxP3 antibody at a concentration of 1 µg per $1x10^6$ cells for 30 min at 4°C. Fluorescence Minus One tubes were analyzed as controls to determine thresholds and single staining tubes to perform color compensation. Flow cytometric analysis was done with a BD Accuri C6 flow cytometer and Cflow software (BD, Belgium, Erembodegem). Analysis was performed on the permeabilized lymphocyte population based on forward and side scatter characteristics. The percentage of Tregs was calculated as the $CD4^+CD25^+FoxP3^+$ cells in the overall $CD4^+$ T cell population.

3.10 IL-12 receptor activation on peripheral blood mononuclear cells

No canine antibody for IL-12 receptor β2 (IL-12Rβ2) was available, thus an antibody for human IL-12Rβ2 was used instead. Canine IL-12Rβ2 has a high homology with human IL-12Rβ2: 85.4% homology at the nucleotide level and 76.8% homology at the amino acid level and well conserved structural motifs.[364] One hundred thousand PBMCs in a volume of 100 µL of FACS staining buffer (BD Biosciences) were used for immunostaining. Cells were stained for the surface antigen IL-12Rβ2, using Alexa Fluor 488-conjugated anti-human IL-12Rβ2 antibodies (R&D Systems, FAB1959G-025, UK). Surface staining was done at 4°C for 30 min. Cells were then washed to remove unbound surface antibodies. Flow cytometric analysis was done with a BD Accuri C6 flow cytometer and Cflow software (BD, Belgium, Erembodegem). Unstained canine PBMCs served as negative controls, whereas stained human PBMCs served as positive controls.

3.11 Immunohistopathology

One cylindrical tumor biopsy of 2x8 mm was taken on day 1, 10 and 15 or 1, 15 and 35 at the identical area in 2 dogs (dogs 4 and 6 respectively). Samples were fixated for 24 h in formol, then rinsed and kept in distillated water until processing. Samples were paraffin-embedded and sections were cut longitudinal. Paraffin-embedded sections were pretreated for antigen retrieval by heating in a microwave for 30 min in a citrate buffer (pH 6.0), and allowed to cool for 20 min. Afterward, endogenous peroxidase activity was blocked by resting during 5 min in a 0.03% H_2O_2 solution (Dako, K4011). The section was rinsed with distillated water and washed once with wash buffer. Then, the section was incubated during 30 min with a 1/100 dilution (in primary antibody diluent Dako, S302283) of cross-reacting primary antibody polyclonal rabbit anti-human T cell, CD3 (Dako Ref.A0452) or polyclonal rabbit anti-human B cell, CD20 (Thermo Scientific, RB-9013-P). The section was rinsed again with wash buffer and incubated during 30 min with the secondary antibody conjugated with peroxidase (Envision Link Rabbit (Dako, K4011)). The section was rinsed twice with wash buffer afterward and incubated during 5 min with 3,3′-diaminobenzidine solution (Dako, K4011). Then, the section was rinsed with distillated water and counter-coloured with hematoxyline, dehydrated and mounted on a slide. Positive controls for CD3 and CD20 stainings were performed on a LN. Photomicrographs were obtained using a Leica microscope DMLB2 with Leica camera type DFC 320 R2 and software program LAS (Leica Application Suite) version 4.0.0 (build:877) (Leica, Belgium).

3.12 Statistical Analysis

All data were analyzed with the statistical software program SPSS (version 19.0). The parametric data were analyzed with the paired sample t-test, the non-parametric data with the Wilcoxon rank-sum test with Bonferroni correction. Statistical significance was determined at $p \leq 0.05$.

4 Results

4.1 Local and systemic release of immune activation-linked cytokines (ELISA)

After the IL-12-CP treatment a significant but transient increase of hIL-12 was measured in tumor lysate (p= 0.046) and serum (p= 0.028) (*Figure 25A, B*). No significant changes for intratumoral IFNγ or serum IL-10 were measured (*Figure 25C, D*), whereas no changes at all for intratumoral IL-10 or serum IFNγ were measured.

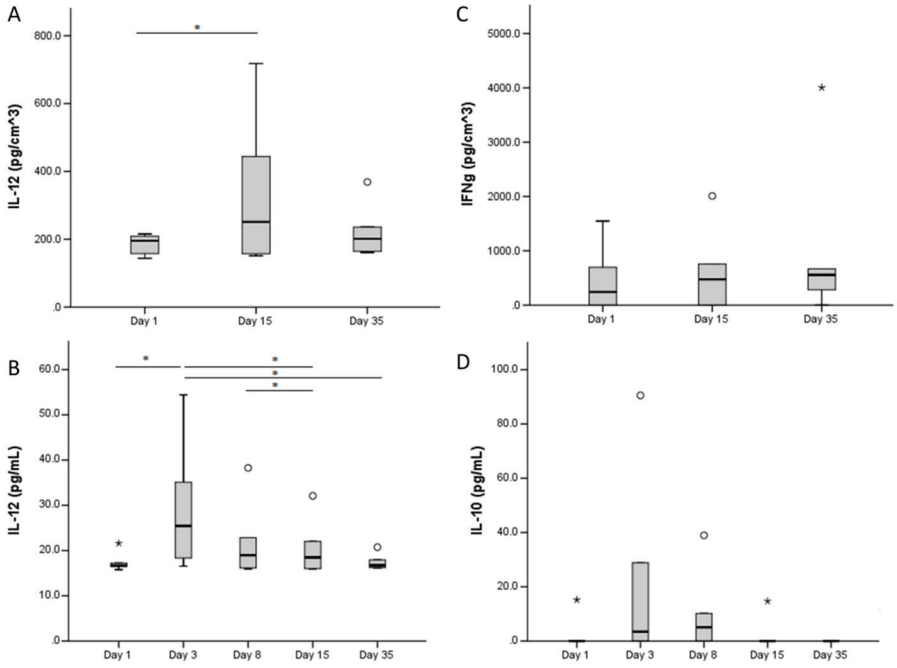


*FIGURE 25. Intratumoral levels of IL-12 (A), IFNγ (C) and serum levels of IL-12 (B), IL-10 (D) during IL-12-CP treatment. ° indicates a mild outlier, * indicates a strong outlier, an underlined * indicates p≤ 0.05 between the different time points (n=6).*

4.2 Complete blood count and biochemistry

After the first hIL-12 EGT treatment, no significant changes were observed for lymphocytes, eosinophils and monocytes at any of the time points, whereas a significant increase in basophils above baseline was noticed at day 8 and 15 (p= 0.043) (*Figure 27, Figure 28*). A significant increase in neutrophil levels was noticed between day 8 and 15 (p= 0.008), but neutrophil levels did not increase above baseline (*Figure 28*). No significant changes were measured for the amount of red blood cells, but a significant increase in reticulocytes was measured at day 8 (p= 0.046) and 15 (p= 0.028) (*Figure 26*). All biochemistry values except SAP remained within range and no clinically relevant changes were detected throughout the treatment cycle. In 2 dogs with bone-infiltrating tumors (dogs 5 and 6), the serum SAP levels exceeded reference levels at day 1 and were decreased at day 3. The level of SAP temporarily decreased after treatment in dog 5 and remained decreased in dog 6.

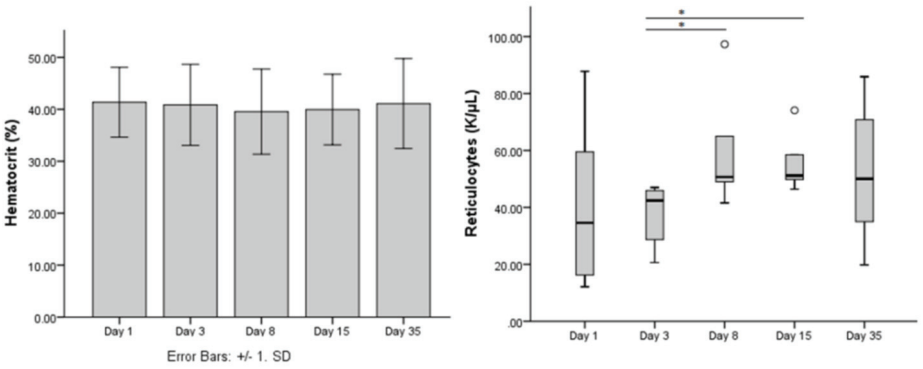


*FIGURE 26. Temporary decrease in hematocrit and increase in reticulocyte response during IL-12-CP treatment. ° indicates a mild outlier, an underlined * indicates p≤ 0.05 between the different time points (n=6).*

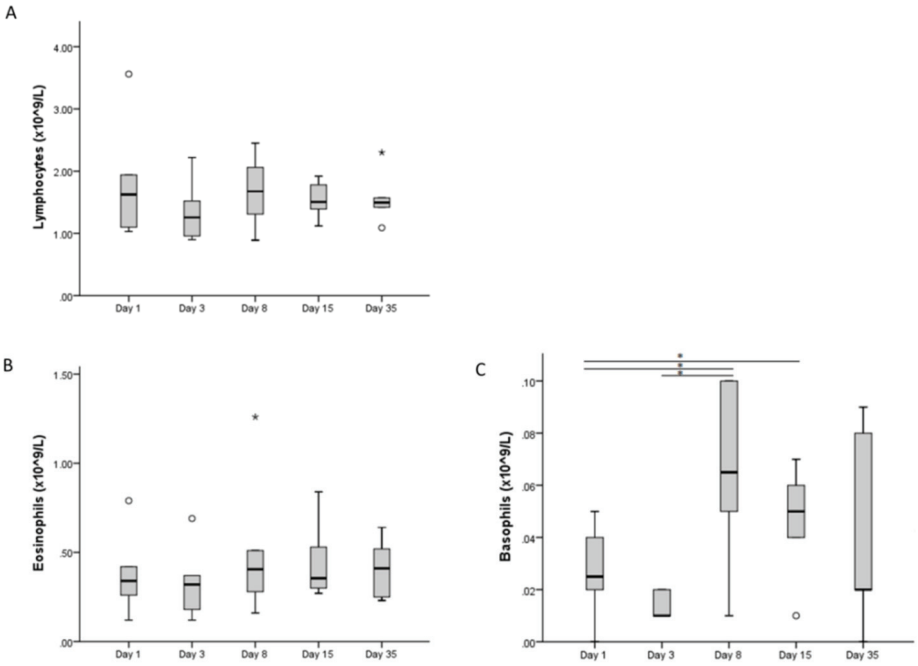


*FIGURE 27. Temporary decrease in the amount of peripheral lymphocytes (A), eosinophils (B) and basophils (C) during IL-12-CP treatment. ° indicates a mild outlier, * indicates a strong outlier, an underlined * indicates p≤ 0.05 between the different time points (n=6).*

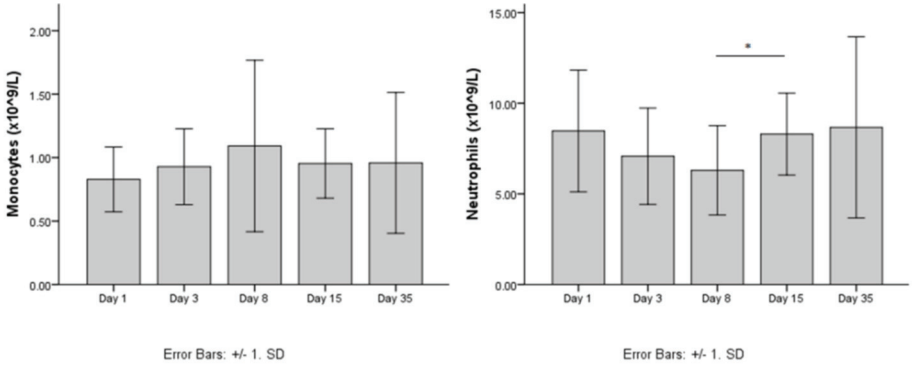


*FIGURE 28. Temporary increase in amount of peripheral monocytes and neutrophils during IL-12-CP treatment. *, p≤ 0.05 between the different time points (n=6).*

4.3 Physical examination findings and side effects after IL-12-CP treatment

Generally, erythema and swelling were apparent 2-3 days after IL-12 EGT treatment (*Figure 29*). The VCOG-CTCAE criteria for adverse effects[326] associated with IL-12 toxicity were used to evaluate the findings of these examinations and are listed in *Table 13*. During electroporation, clear muscle contractions were present which required appropriate sedation. Dog 4 received a second IL-12-CP treatment 5 months after the first IL-12-CP cycle (*Figure 30*) and the observed tumor swelling and erythema were similar to those after the first IL-12-CP cycle.

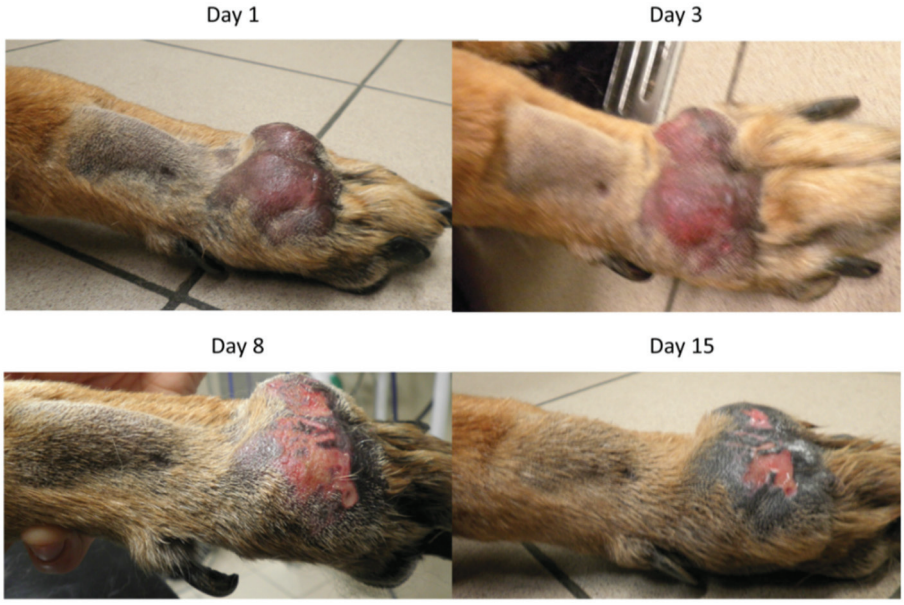


FIGURE 29. Tumor inflammation dorsal to carpus during the first IL-12-CP treatment cycle in dog 4. The tumor inflammation is recognized by erythema on day 3 and swelling on day 8. Photos were taken on day 1 (before treatment) and day 3, 8 and 15 of the IL-12-CP treatment cycle.

FIGURE 30. Tumor inflammation dorsal to carpus during the second IL-12-CP treatment cycle in dog 4. The tumor inflammation is recognized by swelling on day 15 (during). Photos were taken 3 weeks before treatment and on day 15 and 45 of the second IL-12-CP treatment cycle.

TABLE 13. VCOG-CTCAE criteria for adverse effects[326] associated with IL-12 and/or CP toxicity

	Allergic/ Immunological event	Blood/bone marrow			Uro-genital	Constitutional clinical signs			Gastrointestinal			Tumor Pain
Dog	Auto-immune disorder	Neutropenia	Neutrophilia	Thrombo-cytopenia	Cystitis	Fatigue	Fever	Weight loss	Anorexia	Diarrhea	Vomiting	
1	-	-	-	-	-	-	-	1*	-	-	-	-
2	-	-	-	-	-	-	-	1*	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	1*	1	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	2
6	-	-	-	-	-	-	-	1*	-	-	-	-

Auto-immune disorder grade 1 indicates a mild auto-immune reaction. Cystitis grade 1 indicates asymptomatic cystitis; grade 5 indicates death as a consequence of cystitis. Weight loss grade 1

*indicates <10% from baseline; grade 5 indicates death as a consequence of weight loss. Anorexia grade 1 indicates coaxing or dietary change required to maintain appetite; grade 5 indicates death as a consequence of anorexia. Tumor pain grade 1 indicates mild pain not interfering with function; grade 2 indicates moderate pain manageable with standard oral NSAIDs or opiates; grade 4 indicates disabling or uncontrollable pain. * indicates initial weight loss, followed 4 weeks later by complete recovery or weight gain.*

4.4 Clinical response to IL-12-CP treatment

Despite treatment, all primary tumors continued to enlarge. However, the tumor progression was visibly slower after treatment than before in 3 out of 6 dogs. Whereas before treatment the enrolled dogs gradually lost weight, the body weight of 2 dogs, remained stable whereas it increased between 4.2 and 17.4% in the remaining 4 dogs (*Table 14*).

TABLE 14. Effects on patient weight after IL-12-CP treatment

Dog	Weight (kg) on Day 1	Weight gain (kg)	Weight gain (%)
1	27.3	1.2 (Day 35)	+ 4.4
2	31.7	0 (Day 35)	0 (stable weight)
3	23.2	2.3 (Day 35)	+ 9.2
4	32.8	5.7 (Day 90)	+ 17.4
5	14.5	0.6 (Day 35)	+ 4.2
6	21.6	0 (Day 35)	0 (stable weight)
4*	35.8	4.7 (Day 60)	+ 13.1

** Five months after a first treatment cycle, dog 4 was treated a second time.*

4.5 Effects of IL-12-CP on anti-neoangiogenesis

The anti-angiogenic properties of IL-12-CP treatment were measured with CEUS of the tumor in 5 out of 6 dogs (*Figure 31*, *Figure 32*) and the amount of VEGF and TSP-1 was measured by ELISA in serum and tumor tissue in all dogs (*Figure 33*). A significant and

persistent decrease in relative blood volume (characterized by the peak enhancement (PE)) and blood flow speed (characterized by the time to peak (TTP)) was present at day 35 of treatment (p= 0.043 for PE and TTP) in all 5 dogs after IL-12-CP treatment (*Figure 32*). Four out of 5 dogs demonstrated a ≥ 40% decrease in relative blood volume (an indicator of increased survival)[323] at day 35. A non-significant increase in relative blood volume was measured at day 8. A significant increase of intratumoral TSP-1 at day 35 (p= 0.046) coincided with a non-significant decrease in intratumoral VEGF. Interestingly, serum levels of TSP-1 were significantly decreased at day 8 (p= 0.046), which coincided with the lowest amount of serum VEGF. On day 15, serum TSP-1 levels had again significantly increased (p= 0.043) (*Figure 33*).

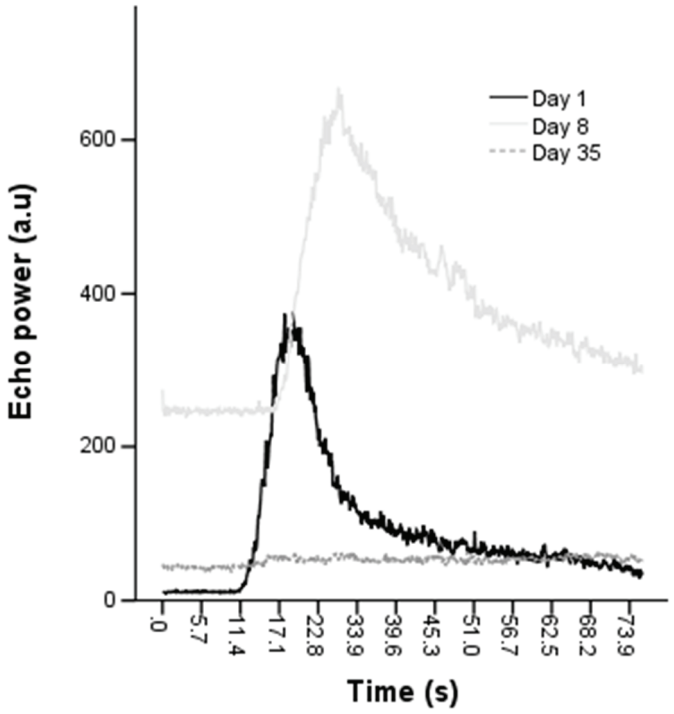


FIGURE 31. Representative time intensity curves after IL-12-CP treatment in dog 2.

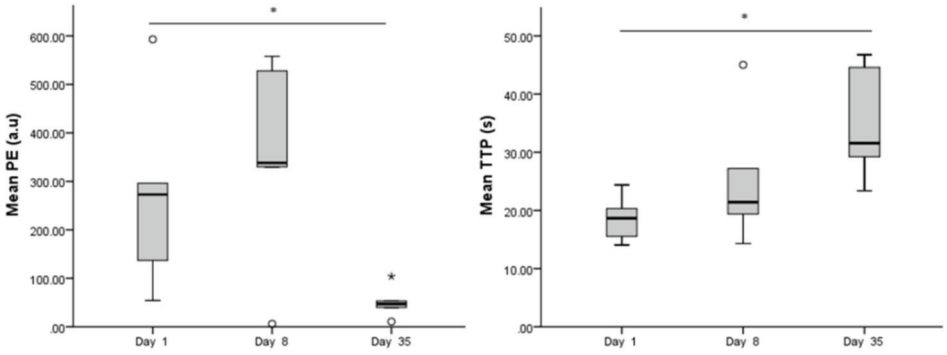


*FIGURE 32. Follow-up of relative blood volume (PE) and blood flow speed (TTP) during IL-12-CP treatment. ° indicates a mild outlier, * indicates a strong outlier, an underlined * indicates p≤ 0.05 between the different time points (n=5).*

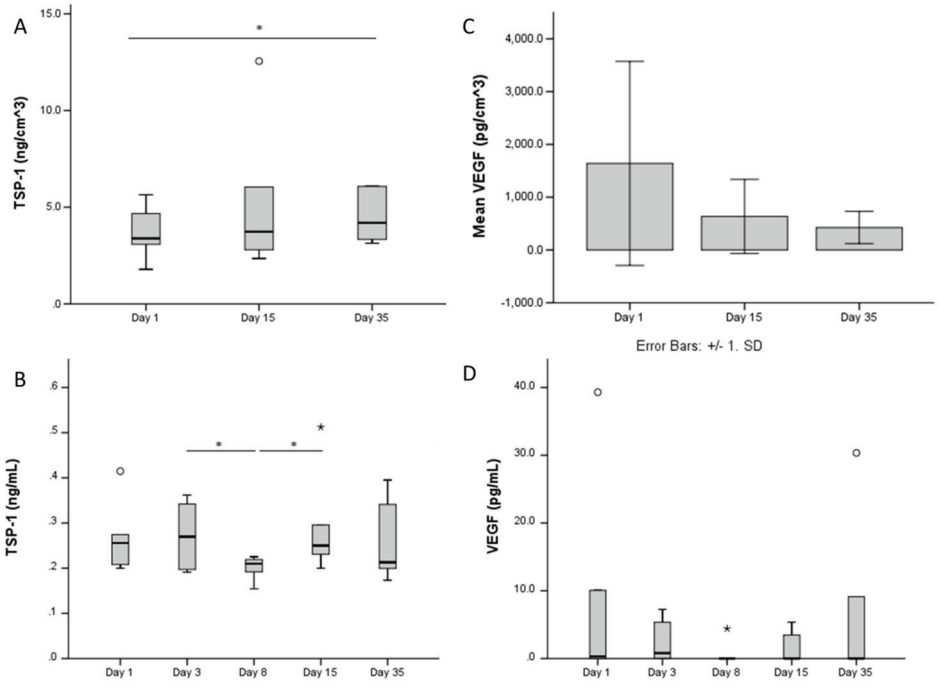


*FIGURE 33. Intratumoral levels of TSP-1 (A), VEGF (C) and serum levels of TSP-1 (B), VEGF (D) during IL-12-CP treatment. ° indicates a mild outlier, * indicates a strong outlier, an underlined * indicates p≤ 0.05 between the different time points (n=6).*

4.6 Effect of IL-12-CP treatment on regulatory T cells

A significant decrease of Tregs was noted in patients treated for 2 weeks with IL-12-CP (3.05 +/-1.52% vs 1.08 +/-0.82%, p=0.046). The percentages of Tregs did slightly increase after day 15, but were still significantly below baseline at day 35 (3.05 +/-1.52% vs 1.43 +/-0.77%, p=0.043) (*Figure 34*).

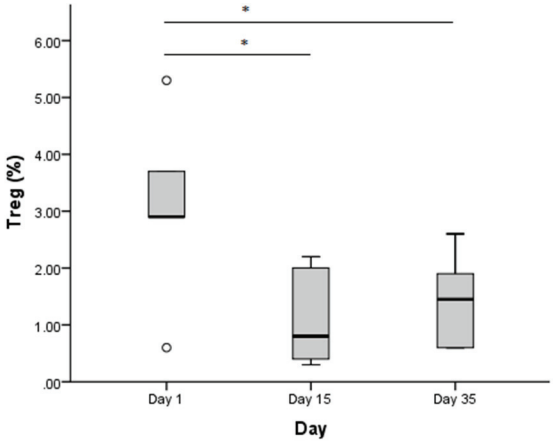


*FIGURE 34. Percentages of Tregs during IL-12-CP treatment. ° indicates a mild outlier, an underlined * indicates p≤ 0.05 between the different time points (n=6).*

4.7 Effect of IL-12-CP treatment on IL-12Rβ2 expression on peripheral blood mononuclear cells

A non-significant decrease of IL-12Rβ2 on PBMCs is noted on day 15 and 35 of the IL-12-CP treatment cycle (*Figure 35*).

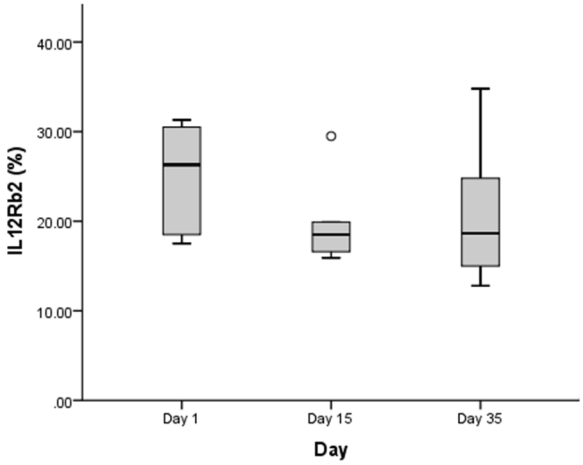


FIGURE 35. Percentages of IL-12Rβ2-positive PBMCs during IL-12-CP treatment. No significant differences were present between the different time points (n=6).

4.8 Effect of IL-12-CP treatment on tumor immune cell infiltration

Tumor biopsies were taken from 2 dogs (dog 4 and dog 6) to evaluate the presence of immune cell infiltration following IL-12-CP treatment. An increase in the amount of T cells was noted on day 15 for both dogs. In contrast, whereas a clear increase in B cells was present on day 15 in dog 4, this was not present until day 35 in dog 6 (*Figure 36*).

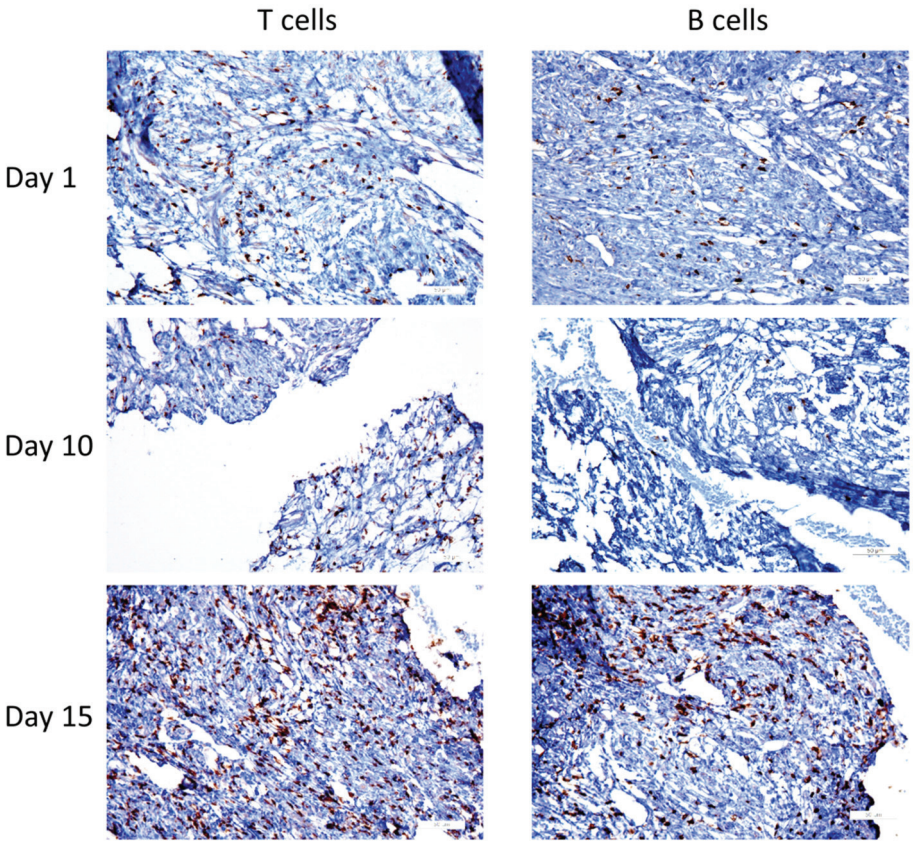


FIGURE 36. Immunohistochemical stainings for T (CD3) and B cell (CD20) tumor infiltration counterstained with haematoxylin (GILL) during IL-12-CP treatment in dog 4.

5 Discussion

We evaluated the anti-angiogenic and immunological effects of the combination of intratumoral hIL-12 EGT with oral metronomic CP.

A remarkable increase in intratumoral relative blood volume was noted 1 week after the first IL-12-CP treatment, followed by a clear decrease in blood volume below baseline on day 35. This transient increase in blood volume may seem paradoxical for an anti-angiogenic treatment, but is a well-known phenomenon in anti-angiogenic approaches,

namely "vascular normalization". Indeed, anti-angiogenic treatments remold intratumoral blood vessels into more mature and functional blood vessels. This normalization decreases the intratumoral vascular permeability and thus the interstitial fluid pressure, leading to a more homogenous perfusion.[365] A decrease in interstitial fluid pressure was confirmed in our study, as all the dogs demonstrated a softer tumor consistency as from 1 week after the first intratumoral hIL-12 EGT.

An important advantage of vascular normalization is the restoration of overall perfusion which reverses the intratumoral hypoxia and low pH. Since hypoxia and decreased pH compromise the cytotoxic functions of immune cells, the improved perfusion will not only allow immune cells to reach the entire tumor but also to display an improved cytotoxic function.[366] The onset of normalization is typically 2 days after initiation of an anti-angiogenic treatment.[365] The normalization window depends on the anti-angiogenic treatment and has been reported to last 2-4[367] or even 2-8 days.[368] Erythema and swelling of the tumor was seen in all dogs 2 days after the first intratumoral hIL-12 EGT. In 2 dogs, the tumor location was prone to accidental self-injury. The tumors of these 2 dogs bled very easily during the first 14 days after treatment. After these first 14 days, the sensitivity to bleeding after accidental self-injury was significantly reduced. These findings reinforce the concept of temporary normalization of the tumor vascularization, associated with cell influx and inflammation, followed by anti-angiogenesis.

Malignant and benign tumors are characterized by a rapid and slow increase of contrast uptake respectively.[345] At day 8, the CEUS registered a non-significant decrease in blood flow speed, which could indicate a less aggressive behavior of the tumor after IL-12-CP treatment. At day 35, the tumor blood flow was significantly slower than on day 1 and the relative blood volume had significantly decreased as well. A decrease in tumor blood volume does not preclude efficient perfusion. Despite a significant drop in tumor blood volume, the delivery of large molecules or immune cells can still be more efficient than before treatment through the remaining vessels.[369] Additionally, a ≥ 40% decrease in relative blood volume was measured in 4 out of 5 dogs. Both tumor vascular normalization[365] and a ≥ 40% decrease in relative blood volume[323] correlated with improved patient outcome. Although the tumors of all treated dogs continued to

progress, it is thus likely that the IL-12-CP treatment induced a slower tumor progression.

In our study, a clear inflammatory response was present 2 days after treatment initiation. As IL-12 upregulates intratumoral vascular adhesion molecules,[213, 327, 332, 333, 334] normalization due to the anti-angiogenic properties of metronomic CP and IL-12 can greatly enhance the overall immune cell infiltration. The observed inflammatory response was probably even further facilitated by the metronomic CP-induced selective depletion of circulating immunosuppressive Tregs. The greater decrease in circulating Tregs measured in our study on day 15 than on day 35 can be explained by the ability of IL-12 to temporarily (minimally 2 weeks) decrease the amount of Tregs.[335] Alternatively, the vascular normalization may have contributed to a more efficient intratumoral deposit of CP and thus a greater decrease in Tregs.

Metronomic CP is known to induce TSP-1 in cancer cells and perivascular cells[370] and TSP-1 inhibits angiogenesis and inflammatory responses in order to suppress tumor formation.[371] In our study, the intratumoral decrease of VEGF-levels logically corresponded with an increase in TSP-1. However, while serum VEGF was lowest on day 8, so was serum TSP-1. Interestingly, the effects of TSP-1 can differ according to the bound cell type.[372] Whereas TSP-1 exerts anti-angiogenic effects on endothelial cells, it is a negative regulator of DC activation[373] which impedes antitumoral immunotherapy. On the one hand, metronomic CP is known to induce TSP-1 and TSP-1 suppresses immune cells via interaction with CD47 on immune cells.[373] On the other hand, metronomic CP has been associated with increased expression of DC maturation markers via Treg depletion in mice.[374] It is possible that the immune activation caused by IL-12-CP treatment induced a negative feedback loop on TSP-1 serum levels.[373] The low amount of serum TSP-1 on day 8 may thus have been the result of an overall immune stimulation and beneficial for an effective immune activation of monocytes, which were at the highest levels on day 8. A decrease in intratumoral VEGF-levels fuels any antitumoral effects even more, as it improves the maturation and function of DCs and reduces Treg and MDSC infiltration.[375, 376] The decrease in VEGF-levels observed in our study could be attributed to IFNγ cascade product IP-10 and the

metronomic CP TSP-1-induced blocking of CD47.[210, 371] Another explanation for the decrease in VEGF is through the observed decrease in neutrophils on day 8. Neutrophils are able to induce VEGF production in cancer cells and a decrease in neutrophils was present on day 8. Although the amount of circulating neutrophils was increased on day 15, the anti-angiogenic effects of the IL-12-CP treatment were maintained. This persistent anti-angiogenic effect can be the result of a change in the neutrophil behavior. Since IL-12 is a powerful stimulator of an antitumoral immune response and the antitumoral activity of tumor-infiltrated neutrophils depends on its microenvironment,[181] it is possible that IL-12 induced a shift in neutrophil behavior. As a consequence, the tumor may not have been able to recruit immature protumoral neutrophils. Therefore, it would be interesting to characterize the maturity of the neutrophils after IL-12 treatments.

Several beneficial developments were noted during IL-12-CP treatment. Most remarkable was the apparent anticachectic effect of the IL-12-CP treatment obtained in 4 out of 6 treated dogs; the body weight remained stable in the 2 remaining cancer-bearing dogs. What is more, the positive effect on the body weight could be reproduced in 1 dog that was treated a second time with intratumoral hIL-12 pDNA after once more gradually losing weight 4 months after the first IL-12-CP treatment.after the first IL-12-CP treatment. Furthermore, bone destruction was evident in 2 dogs (dogs 5 and 6) via radiographs and the measurement of SAP, which is an enzyme for bone destruction and liver disease.[377] The liver function in these 2 dogs was normal and their increased levels of SAP were most likely due to bone destruction. The level of SAP temporarily decreased after treatment in dog 5 and remained decreased in dog 6, possibly indicating a stabilization of bone destruction. Another interesting observation made, was the improved healing capacity of the tumors. Whereas tumors are described as wounds that do not heal,[378] the wounds on the tumors of dogs 2 and 4 did heal after IL-12-CP treatment. Finally, before dog 4 received a second treatment cycle, an unmistakable necrotic odor and deposit were present, which subsided within 2 weeks after the treatment initiation.

Overall, the IL-12-CP treatment seemed to induce a less aggressive and more stable tumor microenvironment. Similar results were seen for the combination of intratumoral IL-12 EGT and oral metronomic CP in cancer-bearing mice.[293] The combination treatment resulted in a significantly enhanced survival of cancer-bearing mice.

To measure the immune activation after IL-12 therapy, one could measure increases in IFNγ secretion and/or IL-12Rβ2 expression.[227] The non-significant increase in intratumoral IFNγ indicates that the hIL-12 was able to stimulate canine immune cells. However, IFNγ may not be a good indicator for immune activation. The amount of IFNγ produced after stimulation by IL-12 does not correlate with IL-12 efficiency.[379] IFNγ can be produced by NK cells as well as T cells after IL-12 stimulation. In both IL-12 responsive and unresponsive tumors, NK cells will release the same amount of IFNγ, but the T cells in IL-12 responsive tumor types express higher amounts of IL-12Rβ2 and release more IFNγ when stimulated by IL-12 than unresponsive tumors.[379] A non-significant increase of IFNγ was measured in the tumor lysate in our study, but probably none of the tumors were immunogenic. This lack in pre-existing tumor immunogenicity seems to be confirmed by the down-regulation of IL-12Rβ2. The IL-12Rβ2 is expressed in activated (tumor-sensitized), but not naive T cells.[380, 381] It is likely that tumor-sensitized T cells are primary targets of IL-12 during tumor rejection,[379] but only a limited number of cancer-bearing patients dispose of a pre-existing immunity.[331] Re-exposure of Th1 cells to IL-12 results in a strong upregulation of the IL-12Rβ2 even in the absence of antigen stimulation.[382] It has been shown that the combination of CP and IL-12 therapy eradicated established large tumors in mice, but only when tumor-sensitized T cells were present.[379] During our study, instead of an upregulation of IL-12Rβ2, a non-significant down-regulation of IL-12Rβ2 was measured on the patients' PBMCs. This non-significant decrease of IL-12Rβ2 could indicate a lack of reactivation of tumor-sensitized T cells, probably signifying that no pre-existing immune response was present. Interestingly, a down-regulation of IL-12Rβ2 indirectly stimulates an antitumoral immune response by lowering the number and functional maturity of Tregs.[383] In the IL-12-CP treated dogs, both a decrease in IL-12Rβ2 and circulating

Tregs were observed. It is possible that the amount of IL-12 present at the start of the treatment led to a higher IL-12Rβ2 down-regulation on the non-tumor-sensitized T cells and encouraged a greater decrease in Treg number on day 15 than on day 35 of the treatment cycle.

6 Conclusion

In conclusion, the combination of intratumoral hIL-12 EGT and metronomic CP is associated with a slower tumor progression as well as with an improvement in quality of life. Indeed, all dogs experienced weight stabilization or increase during and after the IL-12-CP treatment, whereas all dogs progressively lost weight before treatment initiation. Anti-angiogenic treatments are ideally suited to be combined with immunotherapeutic treatments.[375, 384, 385, 386, 387] The temporary normalization as observed with the IL-12-CP treatment of the tumor vasculature facilitates not only drug delivery (such as CP),[359] but also immune cell influx.[365]

Despite the significant drop in tumor blood volume at day 35, the primary tumors continued to progress. Intrinsic cancer cell resistance to metronomic CP exists, but tumor vasculature does not seem to develop resistance to long-term metronomic CP therapy.[388] Therefore, the tumor progression could be the consequence of adaptation of the cancer cells to the anti-angiogenic treatment.[389] In order to further improve the IL-12-CP treatment, a cancer vaccine could be administered prior to IL-12-CP treatment. Cancer vaccines elicit specific antitumoral immune responses and are very much suited to complement the effects of IL-12-CP treatment, since eradication of established tumors through IL-12 requires the presence of a pre-existing antitumoral T cell response.[331]

7 Supplementary data on response to treatment per patient

Despite IL-12 pDNA treatment, all primary tumors continued to grow. A decrease in tissue resistance (Ohm) as measured by the electroporator was observed in all dogs. Further tumor specifics are discussed per patient.

Dog 1: The intra-oral amelanocytic melanoma was surgically removed a first time 7 weeks prior to presentation. Within this time the tumor reappeared and had grown to the same size before surgery (6x4 cm). The tumor was surgically removed a second time and the IL-12-CP treatment initiated 3 days after the surgery. At this time, a new mass was present in the parotic gland area and cytology revealed the same cells as the primary tumor. On US, a hypovascularized liver nodule was detected (8x8 cm) and suspected of metastasis (fine needle aspirate cytology was inconclusive). The accessible metastasis and the wound bed of the primary tumor were treated with intratumoral hIL-12 EGT. Erythema and swelling were present 2 days after treatment. The primary tumor did not resume growth, whereas the metastasis continued to grow. The dog maintained a good appetite and although the dog initially lost weight during treatment, it was regained by day 35. This dog suddenly died on day 61. A necropsy has not been performed.

Dog 2: The soft tissue sarcoma was clearly erythematous and warm by day 3 and swollen by day 8. The inflammation distressed the dog, which led to excessive licking and biting in the tumor. Eventually, wounds were present on the tumor, but these wounds completely resolved by application of honey-based cream (Melolin®) and restriction of access. The mass remained stable in size for 2 months after treatment, after which growth slowly resumed. The dog was in generally good shape and no clinically relevant changes occurred during a 6-month follow-up.

Dog 3: In this dog, hIL-12 EGT of the wound bed of the anal sac adenocarcinoma was initiated the day after surgery. One week after surgical excision of the primary tumor and metastectomy this very aggressive adenocarcinoma manifested in the hypogastric

lymph node (3.5 x 3.5 cm). Interestingly, the LN size was unaltered during 3 weeks, after which it continued to grow. The tumor eventually became so large that it compressed the rectum and the urethra which led to euthanasia of the dog on day 120.

Dog 4: This dog beared a fibrosarcoma dorsal to the carpus. Despite a good appetite, the dog gradually lost weight in the 6 months prior to IL-12-CP treatment and demonstrated a goose step on the tumor-bearing paw. After IL-12 treatment, the dog gained 5.7 kg in 3 months and the goose step waned. The fibrosarcoma demonstrated a discernable softer consistency after IL-12-CP treatment. The dog remained in generally good shape. Five months after the first treatment cycle, the dog's tumor was treated a second time on the owners' request since he was again after gradually losing weight. Once again, a positive effect on weight gain (4.7 kg in 2 months) was observed. A discernable decrease in necrotic tissue was observed after the second treatment of the second treatment cycle (*Figure 30*).

Dog 5: After the first intratumoral IL-12 treatment, a mild swelling of the costal osteosarcoma was apparent. Alkaline phosphatase, an enzyme that possibly indicates bone destruction, exceeded the upper limit at day 1, but remained stable for at least 2 weeks (values between 900 and 1100 (ref 23-212) U/L). The dog gained weight and although the tumor kept progressing, his owners mentioned a clear improvement in quality of life. By day 35, the dog gradually weakened and SAP had become unmeasurably high (>2000 U/L). On thoracic radiographs, clear invasion of the thoracic cavity was present. This resulted in approximately 80% loss of the left lung. Due to further deterioration, the dog was euthanized on day 43.

Dog 6: The rostromandibular fibrosarcoma was firm before treatment, but softened by day 8. Erythema and swelling were present on day 8. The dog remained in generally good shape. Alkaline phosphatase slightly exceeded reference limits at the start of the IL-12-CP treatment, but gradually decreased after the first intratumoral hIL-12 EGT and was decreased by 46% at day 35. This dog was euthanized on day 60 due to tumor progression.

GENERAL DISCUSSION

1 The question is not whether to combine immunotherapeutic treatment options or not, but how ...

1.1 Safety, efficacy, practicality and cost of the combination treatments

It is indisputable that the immune system has a major influence on cancer (treatment) outcome and that immunotherapy has the potential to safely tilt the cancer/immune balance toward a cure for cancer. Yet, the correct application of immunotherapy is still a matter of debate. The future of immunotherapy as a major breakthrough in the treatment of cancer lies most certainly in combinatorial treatment strategies.
The overall goal of this PhD was to design a rational combinatorial therapy by targeting the 3 major arms of the immune system. Per immune arm, many components are available, which allows many possible combinations of components that target a different arm of the immune system. A combinatorial treatment with a high likelihood of clinical implementation is to be preferred. Therefore, the combinatorial treatment chosen should not only effectively manipulate each arm of the immune system, but also be safe, practical and cost-effective.

In Chapter 1, autologous whole cancer cell vaccines were investigated as stimulators of the adaptive immune arm since, of all cancer vaccine types, only autologous cancer cells cover the whole cancer antigen spectrum of the patient. This study was performed in mice. Apart from a wide antigenic range, an advantage of using autologous cancer cells is the possible incorporation of protumoral stromal cells into the vaccine, thus expanding the range of the vaccine from cancer cells to their microenvironment as well.[172] Whereas DC vaccines are presumed to be the most powerful cancer vaccine type,[101] they are also laborious, time-consuming and expensive to manufacture. In fact, Provenge®, the only FDA-approved DC-based cancer vaccine, costs over €77,300/treatment course and is thus (almost) exclusively available to the well-insured cancer patients.[390] Dendritic cell-based cancer vaccines are also present on the

veterinary market and their costs (€2,650 /treatment course) are covered by several veterinary health insurance companies in the UK and Germany.[391, 392]

Safety. The administered cancer cell vaccines, based solely on immunogenically killed cancer cells, were shown to be safe when administered prophylactically. No adverse effects were observed besides transient erythema and swelling at the vaccine injection site in all vaccinated mouse groups. This is in agreement with previous challenges in rodent models. In similarity, early data from canine clinical trials so far confirm the safety profile of cancer vaccines. No significant adverse effects were present in any of the vaccinated canine patients, except for 2 dogs (on a total of 845 dogs).[271, 64]

Efficacy. The administered cancer cell vaccines, based solely on immunogenically killed cancer cells, proved to induce an antitumor response that was as effective in preventing tumor growth after cancer cell inoculation as immunogenically killed cancer cells that were co-incubated or fused to DCs in our prophylactic mouse tumor model. With the cancer cell vaccine based on immunogenically killed cancer cells, a cellular immune response was induced within 45 days after vaccination. Hundred days after tumor inoculation (145 days after the first vaccine), a cellular as well as a humoral immune response was present.

Practical. In the mice, the production of a whole cancer cell vaccine based on immunogenically killed cancer cells was relatively easy. Syngeneic cancer cells were available through the use of an EO771 cancer cell line. These cancer cells only required 2 h of incubation in a Mitoxantrone-containing medium and 22 h of rest to become immunogenic and ready to inject. During this PhD research, no clinical trial with this whole cancer cell vaccine was performed in dogs due to time constraints. The manufacture of the vaccine in dogs would entail a sufficient amount of cancer tissue (at least 10 gr)[117, 393] from which the autologous cancer (and stromal) cells should be extracted. The digestion of the tumor tissue to isolate cells present in the tumor tissue can be achieved in 4 h[394] and ICD of cancer cells in 24 h, thus making it possible to incorporate cancer treatment through vaccination in a clinical setting. If insufficient tumor tissue is present, the use of an allogeneic cell line for certain tumor types in dogs can be considered for vaccine manufacture. However, allogeneic cell-based vaccines might be difficult to integrate into non-academic environments, as they require

approval from the federal agency for medicines and health products (FAMHP), whereas treatments with autologous cells do not.

Cost. Although very cheap in mice (less than €20), the cost price to produce a canine whole cancer cell vaccine based on immunogenically killed cancer cells after surgery is probably more than ten times higher, in the range of €200-300 (only taking into account the price of a cancer cell isolation kit and the equipment required for the induction of ICD). However, this amount is likely acceptable to most dog owners and facilitates the incorporation of cancer cell vaccines into routine oncology treatments.

In Chapter 2, IL-12 was investigated as stimulator of the innate immune response arm in pet dogs with spontaneous tumors. Intratumoral hIL-12 pDNA was administered and electroporated into the cancer cells of cancer-bearing dogs (IL-12 study).

Safety. The first 3 dogs were treated with a 1-day interval between treatments, but 1 of these dogs developed a transient immune-mediated anemia and another a fatal thrombocytopenia. Since these symptoms could be related to IL-12 toxicity and peak expression of IL-12 pDNA was seen after 7 days in a preclinical study,[146] the treatment regimen was altered to 3 administrations with a week interval for all later patients. With this new regimen, no adverse effects were observed.

The dosage of IL-12 pDNA used in other intratumoral IL-12 pDNA trials in dogs was between 150 µg and 1 mg[220, 221, 222] and did not induce any side effects. However, in those trials, at least one week interval was left between IL-12 pDNA treatments in dogs with spontaneous cancer. [220, 221, 222, 225] In contrast, in a human trial, 1 mg of IL-12 pDNA was administered on day 1, 5 and 8 and also did not convey any adverse effects. The amount of IL-12 and IFNγ measured in the tumor tissue and serum of IL-12 treated dogs in our study showed that the dogs who developed anemia or thrombocytopenia did not have the highest amount of IFNγ, the cytokine generally assumed responsible for IL-12 toxicity.[213] These results likely indicate that the 1-day interval regimen was safe. Other reported IL-12 toxicity-related adverse effects include stomatitis, vomiting, (bloody) diarrhea, neutropenia, hypoalbuminemia, increase in liver enzymes, increase in kidney values and hypoglycemia,[395] but none of these effects were observed in any of our IL-12 treated patients. Conversely, the observed adverse effects in 2 out of 3 dogs in

the 1-day regimen are well-known paraneoplastic symptoms.[353, 396] Both dogs that developed adverse effects were in a late tumor stage. Both dogs had a low body condition score and an elevated neutrophilic count, which is an indication for paraneoplastic syndrome.[396] It is possible that the observed adverse effects were not the result of IL-12 toxicity, but rather represented an aggravation of a subclinical paraneoplastic syndrome. Assuming the former, it must be borne in mind that IL-12 therapy could represent a risk for grave complications in patients with (sub)clinical paraneoplastic syndromes.

Another possible safety issue is inherent to the delivery method of IL-12 pDNA. Since IL-12 pDNA electroporation requires thorough sedation of the cancer-bearing dogs and most dogs with cancer are older dogs, they are at a higher risk of developing anesthesia-related complications.[397]

A third concern of administrating hIL-12 pDNA to dogs is the possible development of cross-reacting antibodies against cIL-12. In dogs, cross-reacting antibodies between human and canine tyrosinase have been described for a xenogeneic tyrosinase vaccine.[296] In contrast to the tyrosinase vaccine, it was demonstrated that hIL-12 is recognized by canine immune cells[231] without causing a cross-reaction toward canine IL-12.[349] When hIL-12 immunocytokines were administered to dogs, 8/12 dogs had positive blood samples for antibodies against hIL-12,[227] thus indicating that hIL-12 can elicit a xenogeneic immune response. During this PhD, 2 cancer-bearing dogs received 2 hIL-12 treatment cycles with an interval of 6 months between cycles. In both dogs, the treatment induced a similar inflammatory response 2 days after hIL-12 pDNA treatment. In those dogs, the inflammatory response did not seem to be hindered by the capture of hIL-12 by hypothetical canine anti-hIL-12 antibodies.

Overall, intratumoral administration of hIL-12 pDNA via electroporation to cancer-bearing pet dogs can be considered safe if pre-existing auto-immune responses that might be further stimulated are not present.

Efficacy. In all dogs treated with hIL-12 pDNA, an immune response and an anti-angiogenic effect were observed. Although all primary tumors progressed, in 1 dog a decrease in the size of the abdominal LN metastases was measured. However, the anti-metastatic effect, the immune stimulation and anti-angiogenic effects were all transient.

Two positive effects of the IL-12 treatment did persist. Three owners reported an increase in quality-of-life during the IL-12 study: their dogs showed an increased appetite and no longer gnawed or licked their tumor.

Practical. During this PhD, 16 dogs with various tumor types and sizes were anesthetized and treated with hIL-12 pDNA. In human literature, local lidocaine was applied and an analgesic was offered to all patients with metastatic melanoma treated intratumorally with hIL-12 pDNA electroporation, but no anesthesia was given.[219] However, these tumors were very small, treated with a single electroporation and very thin electrodes (0.3 mm needle diameter),[219] whereas the dogs treated in this study had larger tumors, thus requiring multiple electroporations with sturdy needle electrodes (0.7 mm needle diameter). A disadvantage of having to electroporate hIL-12 pDNA into the larger tumors was the fact that is was time-consuming. To allow optimal pain control and proper sedation prior to intratumoral hIL-12 pDNA injection and subsequent electroporation, the dogs needed to be premedicated 30 min prior to the first insertion of electrodes. The duration of the hIL-12 pDNA administration into the patient's tumor depended on the size and structure of the tumor. As the maximal volume that could be covered with electrodes was approximately 2 cm^3, large tumors required many electrode insertions thus contributing to increased treatment duration. Furthermore, the electroporator could only generate pulses in tumors with a tissue resistance above 50 Ohm. While the tissue resistance of the treated tumor's surface well exceeded the 50-Ohm barrier, this was not true for the core of all treated tumors. As a consequence, the electrodes had to be gradually retracted after tissue insertion until the point where tissue resistance exceeded 50 Ohm. This repositioning of the electrode needles added to the procedural time. In conclusion, although electroporation is the most efficient non-viral method to transfect cells with pDNA, it is not very practical in a clinical setting, especially for large tumors with a heterogenous structure.

Cost. In order to legally administer hIL-12 pDNA to cancer-bearing pet dogs, the construct had to be GMP-grade. The purchase of GMP-grade hIL-12 pDNA is extremely costly. The hIL-12 pDNA used in our study was exceptionally available at 0.7% of the normal minimum price. On average, the total cost of 1 treatment cycle consisted of €162 (anesthesia, hIL-12 pDNA and electrode use included). This

treatment option could have been cheaper if the pDNA did not have to be GMP-grade. In a similar clinical trial in dogs in Slovenia,[226] the use of non-GMP-grade pDNA was allowed; no adverse effects were observed.

In Chapter 3, the combinatorial effects of intratumoral hIL-12 EGT and metronomic cyclophosphamide (IL-12-CP) were evaluated. Cyclophoshamide was chosen as chemotherapeutic as our group demonstrated that a dosage of 12.5 mg/m^2 was able to significantly reduce the amount of circulating Tregs in cancer-bearing dogs.[285]

Safety. A previous study conducted in dogs at the Laboratory of Gene Therapy showed that metronomic CP (CP treatment) was safe.[285] Long-term administration of metronomic cyclophosphamide did result in sterile cystitis in 2 out of 30 dogs after 6 weeks and 10 months. Besides sterile cystitis, none of the adverse effects associated with the cytotoxic dose of CP (immunosuppression and gastro-intestinal adverse effects such as vomiting, anorexia and diarrhea) were present during the study. [285] The combination of IL-12 and metronomic CP did not induce any adverse effects.

Efficacy. A persistent significant decrease in Tregs was observed for IL-12-CP study, which is similar to the results of the CP study performed by Denies and colleagues.[285] Interestingly, a stronger decrease in amount of circulating Tregs was apparent at day 15 in the IL-12-CP study than in the CP alone study of Denies and colleagues.[285] After day 15, the amount of circulating Tregs increased in the IL-12-CP study, yet remained significantly lower than before treatment. These kinetics could be explained by the addition of IL-12, since intratumoral IL-12 administration can induce a temporary (minimal 2 weeks) elimination of Tregs.[335]

The anti-angiogenic effects of IL-12 were enhanced by the addition of metronomic CP: a decrease in relative tumor blood volume was still present at day 35, whereas this was not the case for IL-12 alone (*Table 15*). A significant increase in TSP-1 and a non-significant decrease for VEGF was present in the tumor tissue of dogs treated with the IL-12-CP combination. There were no significant differences in serum VEGF kinetics after CP or IL-12-CP treatment, but, a significant decrease and increase of TSP-1 was present in the IL-12-CP study, which was not the case for the CP study. Interestingly, the downregulation of TSP-1 in DCs produces an effective antitumor response that is

opposite to the protumor effects of TSP-1 silencing within cancer cells.[373] Our results suggest that the function of TSP-1 in endothelial cells and DCs may exert opposite effects during cancer treatment. Both the IL-12 and IL-12-CP study induced a transient decrease in lymphocytes, eosinophils, basophils and red blood cells, but these decreases and increases were more pronounced in the IL-12 study. Nevertheless, tumor inflammation after treatment was present in dogs treated with IL-12 and those treated with IL-12-CP. Immunohistopathology performed in the IL-12-CP study demonstrated immune cell infiltration of B and T cells after the first intratumoral hIL-12 EGT.

TABLE 15. An overview of the anti-angiogenic and immunological effects of the CP study, IL-12 study and IL-12-CP study

Parameter	Metronomic CP	IL-12	IL-12-CP
VEGF serum	No change	No change	Transient decrease, but not significant
VEGF lysate	-	No change (decrease for 2/6 dogs)	Decrease, but not significant
TSP-1 serum	No change	-	Significant decrease followed by significant increase
TSP-1 lysate	-	-	Significant increase
Tumor blood volume	-	Significant, but transient decrease	Significant and persistent decrease
Tumor blood flow speed	-	Significant decrease	Significant decrease
Treg	Significant decrease	-	Significant decrease
Leukocytes	-	Significant decrease and increase of lymphocytes, eosinophils and basophils	Decrease, but less pronounced (significant for neutrophils)

Interestingly, in the IL-12-CP study, 1 dog experienced an outspoken anti-cachectic effect after his first treatment (from 33.7 kg to 38.5 kg). Thereafter, its body weight was stable for about 3 months at what point this dog again started to gradually lose weight. When his weight dropped to 35.8 kg, a second IL-12 treatment was administered and resulted once more in an increase of appetite and body weight. In the following 6 weeks, the body weight increased to 39.1 kg.

Practical. The same practical issues for intratumoral administration of the hIL-12 pDNA in the IL-12 study applied for the IL-12-CP study. The administration of metronomic CP is very practical. Once the Endoxan® tablets are redistributed according to the body surface of the patient, the treatment consists of administrating 1 capsule once a day orally. None of the dog owners experienced any difficulties with the administration of the capsules. Metronomic chemotherapy is already implemented in routine veterinary practice today, mostly to patients with no other treatment options available (unpublished survey data). The ideal dosage per tumor type still needs to be determined, but sufficient scientific evidence indicates that metronomic chemotherapy can be beneficial in a subset of patients.[281, 282]

Cost. Metronomic CP is a safe antitumoral treatment of which the costs are negligible in a combinatorial antitumoral treatment. One box of 50x 50 mg Endoxan® tablets is enough to treat a 30 kg dog for 6 months. Should sterile cystitis occur, metronomic doses of chlorambucil and lomustine have also shown to be able to induce antitumoral responses.[283, 284, 290] Chlorambucil can be given as an alternative, but costs 50 times more than cyclophosphamide. Lomustine is not commercially available in Belgium.

1.2 Rational design of combination therapies in immunotherapy

Many single immunotherapies have given promising results, not least ipilimumab (10-15% durable response rate in humans). What is more, these treatment results could potentially vastly improve when incorporated into well-designed combinatorial strategies. However, it is very important to carefully combine immunotherapeutic approaches to establish a safe balance between efficacy and toxicity.[243] Prior to combinatorial treatment all components should be gradually assembled and evaluated.

The timing of the administration of immunotherapeutic components is open to discussion. For the proposed combinatorial treatment in this PhD, one rationale is to administer the intratumoral hIL-12 pDNA after vaccination[331] since IL-12 stimulates activated T cells and vaccination is able to activate effector T cells. Vaccination itself could be rendered more effective through a (1) decrease in immunosuppressive cells such as Treg, (2) an optimized administration schedule and (3) appropriate use of adjuvants. We have proven that selective and persistent decrease in Tregs after daily administration of 12.5 mg/m^2 CP to cancer-bearing pet dogs as observed by Denies and colleagues[285] remains present when combined with intratumoral IL-12 EGT. There is little known about how the administration schedule of whole cancer cell vaccines may impact their clinical efficacy. Daily exogenous antigen immunization with peptide/protein cancer vaccines for several consecutive days (cluster immunization) has proven to be clearly superior to booster immunization with greater intervals.[132] Cluster immunization mimics the kinetics of a viral infection,[77, 132] which could explain its superior immune stimulation properties compared to immunizations with greater intervals. The principle of cluster immunization is likely applicable to whole cancer cell vaccines. Interestingly, an adjuvant is needed to obtain the superior effect of cluster immunization. The choice of the adjuvant should depend on its potential for toxicity and the subset of immune cells it will stimulate. For example, toxicity will be an issue after daily exposure to the adjuvant CpG as it causes damage to secondary lymphoid organs and diminishes CD8+ T-cell responses, whereas poly I:C does not induce such risk.[133] Adjuvant schedules that respect the natural phases of immune induction might sufficiently skew the anticancer immune response in the desired direction. One option is priming with an effector adjuvant such as IL-12 only, followed by a well-timed secondary boost with a memory adjuvant such as CDX-1127, a CD27 agonistic antibody. It is important to administer the memory adjuvant when the memory cells start to develop and require maintenance through sustained protein kinase B activation.[140]

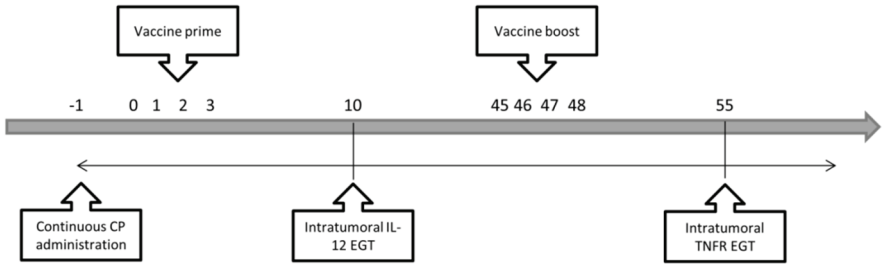


FIGURE 37. Schematic representation of a rational immune combination treatment. Daily metronomic cyclophosphamide is started 1 day prior to the first cluster vaccine (given daily for 4 consecutive days). On day 10, intratumoral IL-12 electrogene therapy (EGT) is administered. A second cluster vaccine is given from day 45-48. On day 55, intratumoral TNFR EGT is administered.

Considering the above, one treatment rationale might be to start metronomic CP 1 day prior to cluster vaccination (*Figure 37*), as low-dose CP given 1 day prior to cancer vaccination significantly enhanced vaccine efficacy in mice and humans.[374, 398, 399] Cluster vaccination (vaccination for 4 consecutive days) is then started, to recreate the effect of a viral infection and optimally stimulate the immune system.[132, 77] Since little time is left between administration of metronomic chemotherapy and the whole cancer cell vaccine, it is conceivable that this regimen will offer a simultaneous multi-targeted approach that inhibits tumor escape. Eight to 15 days after the vaccine administration - when vaccines generally start to generate an immune response[132, 400] this immune response can be enhanced through intratumoral hIL-12 pDNA. IL-12 is known to induce potent differentiation of T cells into effector T cells.[120] Booster vaccinations are most effective when administered after the vaccine has generated a peak immune response. As the peak immune response after peptide cancer vaccines is generally seen 4-10 weeks after vaccination,[400] a whole cancer cell vaccine booster should not be given until 4 to 6 weeks after the first whole cancer cell vaccine cluster vaccination. Cluster vaccination should then be repeated to create a booster effect and encourage the creation of memory T cells. This could be accompanied by the administration of an

immune-memory inducing adjuvant, such as TNFR ligands. TNFR ligands (OX-40, 4-1BB and CD27) activate protein kinase B which directs T-cell differentiation more toward memory T cells than toward primary effector T cells.[120] Many different combination options are available: combinations of various local or systemic immunotherapies as described above, and combinations of local or systemic immunotherapy with standard treatments.

1.3 Influence of biomarkers on the design of combination treatments

Most phase I and II cancer immunotherapy trials in humans have stumbled on 2 crucial issues: how to assess the clinical efficacy of the treatment (*see 1.6*); and how to define the correlates (biomarkers) of clinical efficacy.[145]

While overall survival might be the only objective parameter of clinical efficacy, clinical trials based on overall survival can be exceedingly long and costly, which results in the loss of potentially valuable treatments.[44] One possible solution could be to evaluate overall survival in pet dogs with spontaneous tumors as they have shorter life spans than men. Nevertheless, using the cancer-bearing dog as cancer model is not always feasible. Thus, immunotherapy is in dire need of reliable biomarkers for treatment response, as they could provide information about treatment efficacy during the treatment itself and therefore allow grounded decisions on whether or not to adapt the installed treatment (schedule). Investigating the cancer cell proteome and secretome might lead to the identification of novel biomarkers, which are more effective than the so far evaluated candidates.[44, 401] This search will be facilitated by the worldwide set-up of human (The Cancer Human Biobank from the National Cancer Institute in the US) and veterinary (Uppsala University in Sweden, Cornell Veterinary Biobank in the US, Fischer Bioservices in Maryland in the US, Oncovet in France) biobanks. These biobanks will hopefully help to find appropriate biomarkers for treatment efficiency and common denominators for treatments that are so far only effective in a subset of patients. The combinatorial treatment rationale we propose in 1.2 could be further fine-tuned via the use of predictive biomarkers. Since the proposed cancer vaccine – IL-12 –

CP approach will function via a wide range of effects, it might be useful to focus on these effects to identify potential biomarkers for this approach. Several efficacy parameters were examined during the studies of this PhD. Their suitability as possible biomarkers for the proposed combinatorial cancer vaccine – IL-12 – CP treatment will be discussed below.

1.3.1 Biomarkers for effectiveness of cancer cell vaccines

The whole cancer cell vaccine based on immunogenically killed cancer cells from Chapter 1 induces a cellular and a humoral antitumoral immune response. Although the presence of cancer cell-specific effector T cells or antibodies indicates the ability of the vaccine to initiate an immune response, it cannot predict whether the vaccine will induce a therapeutic effect.[402] However, it has been shown that certain characteristics of vaccine-induced CD8+ T cells can predict the efficacy of vaccines during antitumoral treatment.[402] The formation of (CD62L(-)KLRG1(+) IFNγ+ TNF+) effector-memory T cells after vaccination correlated with a superior antitumoral effect of these vaccines in a therapeutic setting.[402] Thus, if high simultaneous production of IFNγ and TNF by memory T cells is shown after administration of our whole cancer cell vaccine, this could predict a high degree of therapeutic efficacy. Interestingly, the effects of cancer vaccines can be predicted in healthy volunteers,[402] allowing a better estimation of the efficacy potential of the vaccine before administrating it to cancer-bearing patients.
An increase of tumor T cell-infiltration after treatment has also been shown to be associated to responder tumors, whereas a lack of T cell-infiltration after treatment with non-responder tumors.[331]

1.3.2 Biomarkers for effectiveness of IL-12

The main antitumoral activities of IL-12 consist of immune stimulation and anti-angiogenesis. Therefore, it seems logical to evaluate the efficacy of IL-12 treatment by its effect on the immune response (see above) and/or tumor angiogenesis. Although an increase of tumoral and/or plasma IFNγ indicates a response to IL-12, it is not a predictive biomarker for the efficacy of IL-12 treatment.[221, 222, 227] Tumor biopsies for

immunohistopathology were taken from 2 dogs during the IL-12-CP treatment. In both patients, an increase in T and B cell infiltration was present, yet the tumors continued to progress. However, one cannot exclude that these dogs experienced a temporary stabilization or a less aggressive tumor progression than would have been the case when left untreated.

For tumor anti-angiogenesis, multiple biomarker options can be considered. The effect of IL-12 on important angiogenesis pathways such as VEGF can be explored, the perfusion of the treated tumor as well as immune cells with an important role in angiogenesis (such as neutrophils).[401] So far, no reliable predictive biomarkers for the efficacy of anti-angiogenic treatments are available.[401, 403] Although promising in pre-clinical trials, VEGF-inhibition did not yield consistent results in a clinical context.[401] Indeed, many escape mechanisms exist for anti-angiogenic therapy. As a result, a decrease in VEGF does not necessarily mean that the treatment is effective, as cancer cells can rapidly adapt to a new situation by activating alternative angiogenesis pathways to VEGF.[388] Alternatively, instead of measuring markers for angiogenesis pathways, the overall anti-angiogenic effect of the treatment (such as tumor perfusion) can be evaluated through CEUS. CEUS is a new functional validated imaging technique for the prediction of tumor progression.[323] For all treated dogs in the IL-12 study, a transient decrease in relative tumor blood volume was present. This finding might indicate a transient treatment efficacy. In contrast, in the IL-12-CP study, a persistent decrease (of at least 35 days) in relative blood volume was present in 4 out of 5 dogs. In humans, patients treated with several anti-angiogenic agents demonstrated a better overall survival and disease stabilization (free from progression) when a greater than 40% decrease in area under the curve (blood volume) between baseline and day 30 was measured.[323] Furthermore, normalization is associated with a temporary increase in relative blood volume and an improved clinical outcome. In the IL-12-CP study, a temporary increase in relative blood volume was measured with CEUS on day 8. In our IL-12 study, only 2 dogs demonstrated a clear decrease in tumor VEGF, whereas a 40% decrease in area under the curve between baseline and day 35 was present in 7 out of 8 dogs. In our IL-12-CP study, 4 out of 6 dogs demonstrated a clear decrease in tumor VEGF, whereas a 40% decrease in area under the curve between baseline and day 35

was present in 4 out of 5 dogs. Although tumor progression was eventually apparent in every treated dog, these results indicate a temporary stabilization of the tumor progression.

Neutrophil infiltration in advanced tumors is associated with poor clinical outcome. Neutrophils indirectly contribute to tumor progression by inducing VEGF production in cancer cells, recruiting Tregs into the tumor and promoting detachment and evasion of cancer cells.[181] Therefore, the amount of circulating and tumor-infiltrating neutrophils during anti-angiogenic treatment could be a valuable prognostic factor for treatment efficacy. Indeed, in human patients treated with anti-angiogenic drugs, serum neutrophil levels higher than the upper reference limit were shown to be an independent predictor of short-term survival.[404] More specifically, the quantification of circulating immature (TAN2) neutrophils may indicate when the tumor regains its capacity to recruit immunosuppressive cells, rendering the therapy no longer effective. There is a possible correlation between the proportion of neutrophils and lymphocytes and cancer progression.[405] It was shown that animals with head and neck tumors had high neutrophil/lymphocyte (N/L) proportions in their blood. If treatment was successful, a sustained decrease of this proportion was apparent. It is conceivable that the N/L proportion reveals when the treatment is no longer effective and this finding could aid in the creation of an individualized treatment schedule. In 4 out of 6 dogs in the IL-12 study and in half of the dogs in the IL-12-CP study respectively, the N/L proportion at day 35 was lower than that at baseline. In those patients, this finding might reflect that the tumors progressed more slowly than before treatment, but they nevertheless progressed.

In our IL-12 study, 2 out of 9 dogs were presented with distant metastases. One dog died before we were able to evaluate the effect of the treatment on the metastasis, but the remaining dog with distant metastases lived for 210 days after treatment initiation. This dog (with abdominal metastases) demonstrated a transient partial regression of these metastases after administration of hIL-12 pDNA in the primary tumor. In this dog, a decreased amount of VEGF was present in the tumor tissue 1 week after IL-12 treatment and VEGF was still lower than baseline 35 days after the treatment initiation. A decreased tumor perfusion could be demonstrated 1 week after IL-12 treatment in

this dog, and was still lower than baseline at day 35 after treatment initiation, yet higher than at day 8. Circulating neutrophil levels transiently increased during treatment, but were lower than baseline at day 35 after treatment initiation. A lower than baseline N/L proportion was measured on day 35 after treatment initiation. However, on day 15 the N/L proportion was lower than on day 35, thus possibly indicating the transient effect.

1.3.3 Biomarkers for effectiveness of metronomic cyclophosphamide

The main antitumoral activity of metronomic CP is to alter the tumor microenvironment by selectively decreasing the amount of Tregs and by inducing anti-angiogenesis by up-regulating TSP-1. Increases in TSP-1 lead to inhibition of the VEGF pathway and to induction of endothelial cell apoptosis.[272, 274] As discussed above, the data on VEGF as a predictive biomarker for benefit and response are not conclusive.
A clinical trial with 12.5 mg/m^2 CP was previously conducted by our lab in cancer-bearing pet dogs.[285] That trial showed that the metronomic treatment did not yield any change in the amount of circulating TSP-1 or VEGF in the treated cancer-bearing dogs. The lack of effect of 12.5 mg/m^2 CP on serum TSP-1 should not preclude this marker for evaluation of anti-angiogenic treatment with other doses for metronomic CP or other anti-angiogenic treatments. Indeed, in our IL-12-CP study, a significant decrease and increase in the amount of serum TSP-1 as well as a significant increase in the amount of tumor lysate TSP-1 at day 35 compared to baseline were measured.

1.3.4 Biomarkers for evaluating the combination of whole cancer cell vaccination, intratumoral hIL-12 EGT and metronomic cyclophosphamide

In conclusion, despite promising preliminary results, no general or cancer-specific biomarker has yet emerged that could help select patients with a positive prognosis for anti-angiogenic therapy.[401] Lassau and colleagues demonstrated a significant association between a decrease in tumor blood volume and stabilization of tumor progression,[323] but anti-angiogenic effects do not necessarily predict cure rates. On the other hand, if the patient does not have a 40% decrease in tumor blood volume 30 days

after anti-angiogenic treatment initiation, this patient should probably switch to another treatment option.

Based on our findings one could propose to evaluate the treatment efficacy of the combinatorial treatment by measuring high simultaneous production of IFNγ and TNF by cancer-specific memory T cells and a sustained decrease in tumor perfusion and in N/L proportion.

1.4 The influence of patient selection in cancer trials

An important factor in the success rate of clinical studies is the selection of the patient population. In human medicine, only patients who are refractory to established antitumoral treatments can participate in clinical trials. As a result, mostly patients with a cancer in an advanced stage, that has become resistant to most common therapies, are recruited in clinical studies for novel antitumoral approaches. The efficacy of novel antitumoral therapeutics is therefore evaluated on a subset of cancer patients who are notoriously difficult to cure. The evaluation of such novel therapeutics may therefore not result in an objective evaluation of the true potential of the drug. Furthermore, due to the advanced stage of the cancer, many patients are weakened and more sensitive to adverse effects. Treatment regulations in human oncology are stringent and can be quite incapacitating. As such, 1 treatment can be favored over another; even if it is not necessarily the best treatment option. For example, ipilimumab has been FDA-approved since 2011 and is the first-line immunotherapeutic for melanoma patients. Nivolumab, an anti-PD-1 antibody, recently received FDA-approval as treatment for advanced melanoma that does not respond to other drugs such as ipilimumab, although nivolumab offers a more acceptable toxicity, response rate and time to response than ipilimumab.[243] Despite the many advantages of anti-PD-1 antibodies, these antibodies cannot be used as first-line treatment for melanoma patients as they are currently approved only as second-line after failure of ipilimumab or BRAF inhibitors in melanoma patients.[243] Nivolumab is currently under review by the FDA for use as a monotherapy for previously untreated patients.[406] While these regulations do not facilitate initiatives to enroll many human patients in combination trials, they do

not apply for canine patients with spontaneous cancer thus highlighting the significant role pet dogs can play in cancer research.

In veterinary medicine, clinical trials in pet dogs are not constrained by traditional phase I, phase II, and phase III trial designs.[407] Therefore, novel agents can be offered to pet dogs before conventional therapies or during the period of minimal residual disease[407] and allow a more objective evaluation of the potency of the novel agent. Pet dogs are able to participate in clinical trials when their owners sign an informed consent and the clinical trial was approved by an ethical committee. As antitumoral treatments are limited in pets, pet owners are highly motivated to seek novel options for management of cancer in their pets. The decision to let their pet participate in a clinical trial is often influenced by the risks associated with this therapy compared to conventional therapy, as well as their expectations for outcomes and reduced costs for care provided by an investigational trial.[407] As a result, clinical studies in pet dogs offer the much-needed opportunity for immunotherapeutic antitumoral therapies to be studied in ideal circumstances. In our studies, most dogs were presented with a recurrent tumor after surgery. It should be noted that for ethical concerns, standard antitumoral therapies with good/better chances of survival for the pet dog were always advised when the owner was able and willing to pursue these standard treatments. Since these pet dogs are patients, the well-being of the pet dog is always the most important factor to be considered and therefore the best treatment options should always be offered.

1.5 Combinations of immunotherapy with standard antitumoral treatments

Patients with high tumor burden are likely to benefit less from immunotherapy. Continuous exposure of effector T cells to antigen will eventually render them tolerant to the antigen. In case of continuous tumor antigen presence, this tolerance results in tumor outgrowth. Interestingly, the T cells regain functional capacities when they are transferred to an antigen-free environment.[408] Elimination of tumor antigen can thus enhance the generation of an effective immune response. This effect can be reproduced

by performing standard antitumoral therapy prior to immunotherapy. Patients in which the tumor burden is reduced or eliminated through surgery or radiotherapy may benefit from a combinatorial approach with immunotherapy. Finocchiaro and colleagues combined immunotherapy with surgery and observed a remarkable increase in efficiency of immunotherapy when applied in a minimal residual disease setting. A complete excision is not always possible due to the tumor location, therefore 2 tumor burden settings were compared: tumors for which the surgeons were able to obtain clean surgical margins, confirmed by histopathology (complete excision) and tumors for which no clean surgical margins could be obtained (partial excision). After surgery, the cancer had less opportunity to reorganize itself as an impenetrable fortress to the immune system. Overall survival of the group with partial excision and immunotherapy was 4 times higher than in the control group that had partial excision only (323 (46-1321) days versus 78 (29-206) days),[147] while overall survival of the group with complete excision and immunotherapy was 7 times higher than in the control group that had complete excision only (704 (99-2251) versus 101 (11-568) days), thus indicating the impact of tumor load.

Next to surgery, the tumor burden can also be reduced via radiotherapy. Radiotherapy combined with immunotherapeutic approaches may increase cancer-cell killing compared with either modality alone.[409] Promising results were obtained in a phase I study of 14 human patients with advanced/metastatic stage hepatoma. Radiation therapy was followed by localized cancer vaccination. Only 10 patients had completed immunologic response evaluation, which resulted in cancer-specific immune responses in 7 out of 10 patients and 2 partial tumor responses.[410]

The combination of chemotherapy and immunotherapy can be beneficial as well. Combinations of chemotherapy and immunotherapy proved to contribute to a better patient outcome than either treatment alone. A phase II trial in patients with stage IIIb/IV non-small-cell lung cancer or extensive-disease small-cell lung cancer evaluated the combination of standard chemotherapy (carboplatin–paclitaxel (CaP)) with ipilimumab. The effects of treatment schedules were assessed as well. The results showed that this combination was reasonably well tolerated, and that a 'phased regimen' in which immunotherapy began after chemotherapy resulted in substantially

improved progression-free survival compared with CaP alone.[411] These results underline the importance of the correct sequencing of combinatorial treatments.

At the moment, various approaches combining immune-checkpoint blockades with local standard antitumoral treatments such as focused radiotherapy and ablative treatment of hepatic metastases are under investigation. These local standard antitumoral approaches induce cancer cell death thus releasing tumor antigens at the tumor site and combined with the necrozing action of immune-checkpoint blockade can stimulate improved uptake of these tumor antigens by APCs.[243]

Combination of several immunotherapies showed synergistic results. For example, combination in a mouse model of metastatic colon carcinoma of IL-15 with antibodies against CTLA-4 and PD-L1 resulted in a significantly increased antitumor activity compared with IL-15 alone or combined with either anti-PD-L1 or anti-CTLA-4.[412]

Immunotherapy can be applied locally or systemically. As has been shown, systemic therapies are more likely to induce serious adverse effects.[213, 413] Much attention goes out to local administration of newly developed immunotherapeutics. The aim of local delivery is to induce antitumor response while limiting systemic auto-immune toxicity. Large-scale clinical trials evaluating these concepts are being considered.[243]

No matter which combinatorial strategy will be chosen, the balancing of efficacy and toxicity will remain a limiting factor.[243]

1.6 Evaluation criteria for immunotherapeutic treatments

Alongside the need for new biomarkers for assessment of treatment efficacy, new clinical evaluation parameters for response to immunotherapeutic treatment are necessary to avoid unjustified/premature switches in treatment strategies. The classic RECIST criteria describe a standardized way to characterize responses to cancer treatment,[414] but were proven not to be appropriate for the evaluation of immunotherapy.[230] Whereas surgery, chemotherapy and radiation offer a quick tumor reduction, immunotherapeutic treatments typically require more time to be effective. For example, in a subset of human patients treated with ipilimumab immune responses were present 12 weeks after treatment[230] and in intratumorally IL-12 pDNA-treated

human patients several clinical responses were apparent in a span of 6-18 months after treatment.[219] Thus, responses to immunotherapy may be overlooked as new lesions can develop while the immune system is building up an antitumoral immune response.[230] Alternative guidelines to RECIST criteria were developed based on the RECIST criteria and propose immune-related response criteria (irRC). In a retrospective study on melanoma patients treated with ipililumab, an additional 10% of patients were identified to have a favorable prognosis via irRC. According to RECIST criteria, (transient appearance of) new lesions or increase in tumor burden represent progressive disease (and may wrongfully classify the response to immunotherapy of a subgroup of patients as progressive disease). According to the irRC criteria, these patterns (response after an increase in total tumor burden and response in the presence of new lesions) can be associated with favorable survival. Error of judgement in response classification could lead to a possibly inappropriate switch in treatment of patients that may benefit from their actual treatment.

2 Limitations and reflections

In this PhD, the rationale was to activate the 3 arms of the immune system to induce an antitumoral response. In the Laboratory of Gene Therapy, the effect of the 3 arms combined was evaluated in mice and considered safe and efficient. Then, our aim was to break down this combinatorial therapy to its individual components and test each component and combination of components for safety and efficacy in cancer-bearing pet dogs. So far, cancer-bearing pet dogs have been treated with metronomic chemotherapy, intratumoral hIL-12 pDNA therapy and the combination of the former. Each component and combination tested until now was considered safe. A decrease in Tregs was present in dogs treated with metronomic CP, immune stimulation and anti-angiogenic effects were present in dogs treated in the IL-12 study. The IL-12-CP study resulted in a significant decrease in Tregs and a persistent decrease in relative tumor blood volume.

The data obtained in **Chapter 1** indicate that vaccination of tumor antigens associated with DCs may not be strictly necessary to elicit an effective antitumoral immune response. This is obviously a bold statement as we researched these effects in only 1 prophylactic cancer rodent model. It would be interesting to evaluate if this statement still remains true when tested in other cancer models and whether the results in a therapeutic setting are similar to those in the prophylactic setting.

For vaccine manufacturing, CC-DC hybrids were magnetically separated from non-hybrid cancer cells through selection of CD11c-positive magnetic particles. As a result, the hybrid vaccines contained CC-DC hybrids but also unhybridized DCs. This may have influenced the results of the hybrid vaccine groups in our study, as administration of DCs has been shown by Kayashima and colleagues to be capable of inducing immune responses in immunosuppressed cancer-bearing mice.[415] The use of a sorter was explored to isolate purely hybrids, but resulted in a 50% cell loss. Together with the long duration of the sorting process, it was deemed unpractical.

As the precise antigenic content of cancer cells is unknown, it is possible that administration of autologous whole cancer cells could induce an immune response mainly targeted against immunodominant non-essential cancer antigens. However, there is still a strong rationale to immunize patients with antigen-rich vaccines (such as whole cancer cell vaccines) as opposed to a single antigen vaccine to prevent tumor escape.[416]

For this study, bone marrow from approximately 4 mice had to be harvested to have enough DCs to vaccinate 11 mice once. Since the co-incubation and fusion groups were vaccinated twice, a minimum of 16 mice had to be sacrificed to enable the vaccination protocol of 22 other mice. Fortunately, in dogs and humans this breach into the 3R-principle is not present. Due to their body size, DCs can be isolated in dogs and humans via leukapheresis.

Electrofusion is known to induce less cell death than PEG-mediated cell fusion,[313] but, in our hands, it proved difficult to standardize and yielded unsatisfactory results. For a reduction in variability, PEG-mediated fusion was chosen. However, the electrofusion option probably needed more optimization and, in the end, may have resulted in higher fusion percentages and thus a more efficient vaccine manufacture.

A potential shortcoming in the design of our whole cancer cell vaccine experiment was the use of xenogeneic serum proteins during cancer cell culture. Splenocytes of naive mice were capable of killing cancer cells during the cytotoxicity test. Their cytotoxicity could be explained by the innate immune response against cancer cells or xenogeneic serum proteins attached to the cancer cells. If an immune response was elicited against the undesired presence of xenogeneic proteins, this clouds the true effects of the cancer cell vaccine itself and the usefulness of the translational study. Therefore, it might have been interesting to research what the effects of the vaccines were if the cancer cells were grown in serum-free medium. In our study, the EO771 cells were grown in a medium containing 10% bovine serum. Bovine serum contains xenogeneic proteins and despite several wash steps before administration of the cancer vaccine, their presence in the cancer vaccine cannot be excluded. Therefore, it is conceivable that the immunogenicity of the cancer vaccines was boosted by the repeated presence of xenogeneic protein in the vaccine groups, whereas the control group only came into contact with the xenogeneic proteins once, namely during tumor inoculation. A possible test could be to twice administer $5x10^5$ non-tumoral cells grown in serum-containing medium to naive mice, with an interval of 35 days. One week after the last "vaccination", $5x10^5$ EO771 cells are administered to these mice. If the tumor develops, this means that the potential presence of serum proteins attached to the vaccine cells will have no impact on tumor outgrowth. If no tumor growth is present, the immune system of the vaccinated mice was trained to recognize xenogeneic serum proteins and killed the inoculated cancer cells in a bystander effect.

In **Chapter 2**, the antitumoral effects of intratumoral hIL-12 EGT were evaluated in cancer-bearing pet dogs. Several alterations could have been made to render the gene transfer of intratumoral hIL-12 pDNA more efficient. The hIL-12 pDNA was diluted in phosphate-buffered saline, whereas 0.45% NaCl saline is one of the best formulations for DNA transfer in muscle cells via electroporation *in vivo*.[417] Furthermore, the properties of the DNA itself can significantly impact the gene transfer efficacy. The size of the DNA affects transfection rates as smaller DNA enters the transfected cell more easily than larger DNA. The pDNA in this study was highly suitable for *in vivo*

transfection. Plasmids can vary in size from 1 to over 200 kbp and the plasmid used in this study was between 5-6 kbp, which can be considered as small-sized DNA.[418] Furthermore, its properties were most suited for high transfection efficiency since it was demonstrated that a vector system with only a few gene cloning sites, a kanamycin-resistant gene, a CMV promoter, and a human growth hormone polyadenylation tail consistently outperformed the transfection efficiency of vectors with multiple cloning sites, an ampicilin-resistant gene, and other types of promoters and polyadenylation sites via electroporation. The hIL-12 pDNA used in our research project had a kanamycin-resistant gene, two separate CMV promoters and a human growth hormone polyadenylation tail. [419] However, a CMV promoter is known to be sensitive to IFNγ-induced attenuation.[357] Since the IL-12 pDNA will induce production of IFNγ, this sensitivity could explain the apparent lack of further expression of hIL-12 after the second intratumoral hIL-12 EGT treatment. As our plasmid contained a CMV-promoter, it is likely that the transgene expression was attenuated once a sufficient amount of IFNγ was built up. This argument is further supported by enhanced transgene expression seen *in vivo* after administration of neutralizing anti-IFNγ antibodies.[338, 357] Another possible but less likely explanation for the relatively short expression of hIL-12 in our studies, could be formation of antibodies against hIL-12. Two dogs were treated twice with the same plasmid; if anti-human antibodies would have been present, the impressive local intratumoral inflammatory responses would not have been observed a second time. Furthermore, the choice for use of supercoiled hIL-12 pDNA may have influenced the duration of the expression as well. Whereas linear pDNA is more suited for stable transfection, supercoiled pDNA will induce transient transfection.[420] A distinct advantage of supercoiled DNA is that it is less vulnerable to degradation by exonucleases as is linear DNA. Thus, the non-integration and subsequent loss of supercoiled pDNA during cell multiplication is highly likely to cause a dilution effect. Finally, the inflammation induced by IL-12 can evoke a compensatory action by the immune system. The immune system always seeks homeostasis: if an inflammation is too intense or too long, it will induce a regulatory effect to contain it.

During the IL-12 studies, the same electroporation parameters were used for every patient. The electroporation protocol used in our IL-12 studies was possibly not

optimal for each individual tumor as the best protocol will differ per tumor type and even per individual tumor due to intratumoral heterogeneity (amount of necrosis, vascularization, …).[233] Electroporation requires therefore a lot of optimization that may not be possible during clinical studies; in addition, reliable, quantitative conclusions can only be derived when a large number of tumors is evaluated.[421] As it was not possible to optimize delivery for every tumor of every treated dog, our protocol was based on BTX's instructions as well as general guidelines from the literature. Currently used field strengths are either high field strength (>700V/cm) or low field strength (<700V/cm) with short pulses (microseconds) or long pulses (milliseconds (msec)). The combination of these variables depends on the target tissue. Generally cancer cells require a low field strength with long pulse, whereas muscle cells require a high field strength with short pulse.[233, 422] In our protocol, cancer cells were therefore pulsed with low field strength and long pulses (2 pulses of 450 V/cm and 8 pulses of 100 V/cm with pulse duration 0.05 msec and 10 msec respectively). So far, the ideal protocol has not been established and more research is needed to clarify the effects of certain electroporation protocols. For example, it is not known why in muscle cells, 1 moderately high voltage pulse (600 V/cm) followed by 4 low voltage pulses (80 V/cm) was found to be more efficient in inducing gene expression than 1 moderately high voltage pulse (600 V/cm) followed by 8 low voltage pulses (80 V/cm).[226] This difference in efficiency could possibly be due to the higher amount of pulses administered in the second protocol. Electric resistance of tumors drops after the first few pulses. The resistance drop is indicative of the breakdown of the skin barrier and/or tissue cell membranes. The less resistance, the more current is evoked by subsequent pulses which lead to cell death.[421] Cell death defeats the purpose of transfection, as living cells are necessary to express the transgene.[421] However, a third electroporation protocol in the study of Pavlin and colleagues with simply 8 low voltage pulses (200 V/cm) demonstrated a gene expression equal to the 1 moderately high voltage pulse followed by 4 low voltage pulses. What is more, the administration of 6 lower voltage pulses (100 V/cm) did not induce any transfection at all.[226] Different electric pulse voltages were also compared in laboratory dogs with CTVT: 200, 500 and 900 V/cm, the pulse duration (50 msec), number of pulses (10 times) and DNA amount (1 mg) were fixed.

The highest expression was measured after 200 V/cm.[146] Those results demonstrate that lower voltages are more likely to induce efficient transgene expression, but it must be borne in mind that these studies were performed with transfection markers luciferase or green fluorescent protein. These markers may not always be representative of the pDNA eventually used in the study, as transfection efficiency also depends on the pDNA characteristics.[419] Nevertheless, during our studies, hIL-12 was measured in the tumor tissue or blood stream of every patient thus indicating that electroporation was efficient in inducing transfection of hIL-12 pDNA into the cancer cells.

Alternatives for electroporation were not explored by our research group, since viral vectors are not considered safe[42] and electroporation is the most efficient non-viral delivery system to date.[422] However, the trauma induced by the needle electrodes and the repeated anesthesia, necessary to effectuate the treatments, are adverse to the patient's immune system as well.[423] The efficiency of other delivery methods may be less dependent from the tumor (type) heterogeneity and may also be more time-efficient. Anesthesia is not required with non-invasive painless transfection methods such as sonoporation, magnetofection or administration of lipoplexes, but so far they demonstrated a lower transfection efficiency.[356, 421, 422, 424] If the transfection efficiency of sonoporation were equivalent to electroporation, sonoporation would have been ideal to use in our study, since the treatment efficacy was already evaluated through CEUS imaging and sonoporation would have required little extra effort. Sonoporation is a safe, noninvasive and flexible site-specific transfection technique. After the incorporation of the pDNA within the microbubbles, these microbubbles are administered intravenously. At the tumor site, ultrasound waves will temporarily permeabilize the cancer cell membranes to allow cellular uptake of pDNA.[425] Sonoporation could also allow targeting of TDLNs and thus reinforcement of the antitumoral immune response. Further research on this alternative delivery method should definitely be pursued to facilitate the translation of gene transfer to the clinic and increase patient compliance. Although upon today lipoplexes represent a less efficient transfection method than electroporation, it is highly stimulating that significant antitumoral results were obtained through gene therapy with lipoplexes.[147]

Per treatment session, 1 mg of hIL-12 pDNA was administered to every dog in the study. The rationale was that efficacy and safety were previously demonstrated with that particular dose.[146, 221] In our studies, the hIL-12 pDNA solution was diluted according to the estimated tumor volume to obtain an even distribution of the hIL-12 pDNA throughout the tumor. Therefore, 1 mg of hIL-12 pDNA solution was diluted to a sixth of the tumor volume prior to administration. However, the dilution of the pDNA may have resulted in a relatively much lower amount of IL-12 producing cancer cells in dogs with large tumors. Indeed, during the IL-12 and IL-12-CP study, the highest intratumoral amounts of hIL-12 were measured in dogs with a relatively small tumor.

It is advisable to evaluate if higher IL-12 pDNA doses are still safe and, if so, to perhaps adapt the hIL-12 pDNA dosage to the amount of tumor tissue (same dosage/cm^3 tumor tissue). However, since differences in tumor organization will impact gene transfer as well, one must always take variability in gene transfer between patients into account.[233]

In future studies with IL-12, it would be advisable to routinely collect blood samples and measure markers of paraneoplastic syndromes, such as auto-immune antibodies, prior and during the IL-12 treatment. In our study, the presence of auto-immune antibodies was confirmed in only one dog with adverse effects (transient anemia). Another dog developed peripheral neuropathy, fatigue and respiratory dysfunction (phased breathing). The peripheral neuropathy could have been the consequence of tumor swelling on the nearby nerve, but peripheral neuropathy and former symptoms have also been described as an expression of a paraneoplastic syndrome such as myasthenia gravis.[396, 426] Measurement of auto-immune antibodies and metabolic imbalances could give us an indication of how likely enrolled patients will develop a paraneoplastic syndrome. Unfortunately, the paraneoplastic syndrome represents a wide range of cancer-associated anomalies that cannot be detected through the presence of one general antibody. One option could be to run a screening test for paraneoplastic symptoms most associated with the selected tumor type.[396, 426]

In **Chapter 3**, a reduced residual disease setting was studied in 2 dogs, as surgery was performed within 4 days prior to the first intratumoral hIL-12 EGT.

In the first dog, the intra-oral amelanocytic melanoma was surgically removed a first time 7 weeks prior to presentation after which the tumor quickly regrew. The tumor was surgically removed a second time and the tumor margins were treated with hIL-12 EGT 3 days later. Although the tumor margins on histopathology were minimal, the resected tumor did not resume growth (whereas it was the case after the first surgery). At the time of the first intratumoral hIL-12 EGT, a new tumoral mass was present in the parotic gland area and cytology revealed the same cell type as the primary tumor. Despite intratumoral hIL-12 EGT, the second mass continued to progress.
In the second dog, removal of a primary anal sac adenocarcinoma and its abdominal LN metastases was followed by hIL-12 EGT of the wound bed of the primary tumor. Although the tumor reoccurred in the remaining hypogastric LNs 1 week after surgical excision of the primary tumor and the metastectomy, the tumor size then remained stable during 3 weeks before it continued to grow.
These results seem to indicate that a reduced disease setting generates a more pronounced and longer equilibrium phase between the immune system and the tumor. Unfortunately, a fully minimal residual disease setting could not be obtained for these dogs, due to the locations of the metastases and/or the cost of the extra surgery.
In our IL-12-CP study, intratumoral hIL-12 EGT was complemented by oral intake of metronomic CP. Although CP (Endoxan®) is a very cheap chemotherapeutic, it is not always easy to implement this treatment option into common veterinary practice. During the IL-12-CP study, this was no issue as we redistributed the Endoxan® tablets ourselves. However, for owners who wanted to continue the metronomic chemotherapy after the IL-12-CP therapy cycle, this proved to be complicated, since only a minority of pharmacists was willing to redistribute the Endoxan® tablets according to the body surface of the cancer-bearing dog.

3 Perspectives of combinations in immunotherapy

Cancer immunotherapy has yielded promising results for a subset of cancer patients. So far, success rates were modest, but not illogical as the relation between the immune system and cancer is complex and the immune system needs help on multiple fronts to

fight the cancer efficiently. Therefore, success rates will most likely increase, as combination therapy is the next big step in immunotherapy. However, more combination therapy will lead to more possible toxic interactions, thus these combinations should be carefully designed and gradually combined to monitor their efficacy as well as their safety.

At the Laboratory for Gene Therapy, the effects of combination treatments will be further evaluated. Other possible antitumoral research avenues of hIL-12 pDNA could include its effects on surgery/anesthesia-induced stress response. As this stress response causes immunodepression and tumor progression is largely mediated by reduced NK cell-activity, IL-12, as a powerful stimulator of NK cells,[427] could potentially inhibit surgery-related stress responses and its associated side effects. It would also be interesting to further investigate the positive effects of IL-12 on quality of life, such as weight gain and the display of a more energetic behavior, in treated patients.

SUMMARY

As long as there has been life, there has been cancer. Various cases of cancer have been detected in fossile remnants of dinosaurs since the Jurassic era.[428] Many avenues for the treatment of cancer have been explored over thousands of years, yet cancer still represents the second leading cause of death in the world after cardiovascular diseases.[429] In the last 150 years, advancements in cancer therapy have exponentially increased as surgery was reinforced by chemotherapy and radiotherapy. Although the seeds of immunotherapy were planted at the end of the nineteeth century, immunotherapy has never been explored with so much vigor as today,[4] as it bears the potential to inhibit cancer recurrence.

While several immunomonotherapies have been FDA-approved and seem very promising,[7] the overall success percentages are low. The interactions between the immune system and cancer are numerous and complex. Cancer cells are able to rapidly adapt to and escape antitumoral treatment. Therefore, it seems logical that when immunotherapy targets only one element in this web of interactions between cancer and the immune system, it will not yield significant results.
Focus should shift from manipulating one to several cancer targets during cancer treatment. Therefore, combination therapy is the next big step in immunotherapy. Although much knowledge has been gained about the safety of monotherapies, this cannot guarantee that combination therapy will not cause toxic or auto-immune responses. The chosen combinations should be carefully designed with regard to possible interactions and treatment schedules. Then, these therapies can be gradually combined, while their efficacy as well as their safety is closely monitored.

For this thesis, several elements of a combination immunotherapy were evaluated for safety and efficacy, whereafter these components were gradually combined and re-evaluated for safety and efficacy. The elements of the combination immunotherapy consisted of the 3 arms of the immune system: (1) the adaptive immune system was stimulated with a cancer vaccine, (2) the innate immune system with IL-12 and (3) the immunosuppressive cancer microenvironment suppressed via metronomic cyclophosphamide. The first arm was evaluated in mice, the second and third in dogs.

Chapter 1 describes the safety and prophylactic efficacy of 3 different whole cancer cell vaccines based on immunogenically killed cancer cells in mice. These 3 different vaccine groups were compared for immunological response and survival. Whole cancer cell vaccines based on immunogenically killed cancer cells were able to induce an antitumoral immune response against tumor outgrowth as effectively as the whole cancer cell vaccines combined with DCs (co-incubated or fused). However, the immune responses responsible for this protection differed between the vaccine types. The immunogenically killed cancer cells without DCs protected the mice against tumor development via a cellular response only. The protection against tumor development in the fusion vaccine occured mainly via a humoral response, whereas the co-incubation vaccine protected the mice via a cellular as well as a humoral immune response. These findings indicate that vaccination with immunogenically killed cancer cells (with or without DCs) may be a useful prophylactic strategy against (recurrent) cancer growth. Furthermore, it proposes that a cancer vaccine design not necessarily has to incorporate DCs as whole cancer cell vaccines consisting solely of immunogenically killed cancer cells were as effective in inhibiting tumor outgrowth. This finding could greatly improve the practicality and cost of cancer vaccines in cancer-bearing pet dogs.

In Chapter 2, the safety and antitumoral effects of intratumoral hIL-12 pDNA on spontaneous tumors were evaluated in pet dogs. Whereas immune stimulation and anti-angiogenic effects on tumor perfusion were present in every treated dog, no cures were obtained. However, 1 dog with metastases of the iliac LNs did experience a transient partial regression in the size of these metastases, while the primary tumor continued to progress. This makes us hopeful that IL-12 could play an important part in anti-metastatic effects. IL-12 is a powerful stimulator of the innate immune system and many innate immune cells are very plastic and exert pro- or antitumoral activities according to their microenvironment. It is very likely that IL-12 effectively influences the transition from a protumoral to an antitumoral microenvironment in less-established tumors such as metastases and certainly metastatic LNs as they harbor immune cells responsive to IL-12.[430]

In Chapter 3, the safety and antitumoral effects of the combination of metronomic cyclophosphamide and hIL-12 pDNA with spontaneous tumors were evaluated in pet dogs. Inflammatory responses were observed in every dog after treatment as well as a clear reduction in tumor perfusion. Whereas in the IL-12 study the blood flow speed within the tumors was transiently decreased, it was persistently decreased after IL-12-CP treatment. Those persistent anti-angiogenic effects indicate that the overall survival of the pet dogs was increased after IL-12-CP treatment despite the absence of cures, which was similar to the findings in the preclinical mouse study. Furthermore, IL-12-CP treatment can play a palliative or supporting role for cancer patients as weight gain or stabilization after progressive weight loss was seen in all IL-12-CP treated pet dogs.

SAMENVATTING

Het ontwikkelen van kanker is van alle tijden. Verschillende gevallen van kanker werden ontdekt in de fossiele resten van dinosaurussen sinds het Juratijdperk.[428] Onderzoek naar kankerbehandelingen wordt al duizenden jaren uitgevoerd, maar toch blijft kanker de tweede grootste doodsoorzaak na cardiovasculaire aandoeningen.[429] Gedurende de laatste 150 jaar nam de vooruitgang in kankerbehandelingen exponentieel toe: zo werd chirurgie vergezeld door chemotherapie en radiotherapie. Hoewel de zaden voor immunotherapie reeds gepland werden aan het eind van de negentiende eeuw, werd het nog nooit zo verwoed onderzocht als vandaag,[4] aangezien het het potentieel biedt om kankerherval te voorkomen.

Verschillende immunomonotherapiën werden goedgekeurd door het FDA en lijken erg veelbelovend,[7] maar de algemene succesratio is laag. Er zijn vele en complexe interacties tussen het immuunsysteem en kanker. Daarbovenop zijn kankercellen in staat om zich snel aan te passen aan antitumorale behandelingen en zodoende te ontsnappen aan de effecten van de kankerbehandeling. Daarom lijkt het logisch dat wanneer slechts één element geviseerd wordt binnen het kluwen aan interacties tussen de kanker en het immuunsysteem, dit weinig significante resultaten zal opleveren.
De focus moet daarom verplaatst worden weg van het manipuleren van één kankerdoelwit naar het manipuleren van meerdere doelwitten gedurende behandeling. Zodoende is combinatietherapie de volgende grote stap in immunotherapie. Hoewel veel kennis verworven werd over de veiligheid van monotherapiën, biedt dit geen garantie dat combinatiebehandelingen geen toxiciteits- of auto-immuunresponsen veroorzaken. De keuze voor bepaalde combinaties moet logisch zijn en rekening houden met mogelijke interacties en behandelingsschema's. Nadien kunnen deze behandelingen gradueel gecombineerd worden, terwijl hun efficiëntie en veiligheid van nabij opgevolgd worden.

Voor deze thesis werden verschillende elementen van een combinatie-immunotherapie geëvalueerd op veiligheid en efficiëntie, waarna deze componenten gradueel werden gecombineerd en opnieuw beoordeeld op veiligheid en efficiëntie. De componenten van de combinatietherapie maakten deel uit van de 3 armen van het immuunsysteem: (1)

het adaptieve immuunsysteem werd gestimuleerd met een kankervaccin, (2) het aangeboren immuunsysteem met IL-12 en (3) de immunosuppressieve kankermicro-omgeving onderdrukt via metronomische cyclofosfamide. De eerste arm werd beoordeeld in muizen, de tweede en derde in honden.

Hoofdstuk 1 beschrijft de veiligheid en profylactische efficiëntie van 3 verschillende volledige kankercelvaccins gebaseerd op immunogeen gedode kankercellen bij muizen. Deze 3 verschillende vaccingroepen werden vergeleken voor immunologische respons en overleving. Volledige kankercelvaccins gebaseerd op immunogeen gedode kankercellen waren in staat om een even efficiënte antitumorale immuunrespons op te wekken tegen tumoruitgroei als de volledige kankercelvaccins die gecombineerd werden met dendritische cellen (gecoïncubeerd of gefusioneerd). De immuunresponsen verantwoordelijk voor deze bescherming verschilden tussen de vaccintypes. De immunologisch gedode kankercellen zonder dendritische cellen beschermden de muizen tegen tumorontwikkeling via enkel een cellulaire respons. De bescherming tegen tumorontwikkeling bij het fusievaccin vond plaats via een humorale respons, terwijl bij het combinatievaccin dit via een cellulaire en humorale respons gebeurde. Deze bevindingen geven aan dat vaccinatie met immunogeen gedode kankercellen (met of zonder dendritische cellen) een bruikbare profylactische strategie kunnen zijn tegen (recurrente) tumorgroei. Verder geven deze resultaten aan dat een kankercelvaccin niet noodzakelijkerwijs dendritische cellen hoeft te bevatten aangezien een volledig kankercelvaccin bestaande uit enkel immunogeen afgedode kankercellen even efficiënt was in het verhinderen van tumorontwikkeling. Deze bevinding kan een grote verbetering betekenen voor de toepasbaarheid en de kost van kankervaccins bij honden met spontane tumoren.

In Hoofdstuk 2, werden de veiligheid en antitumorale effecten van intratumorale afgifte van plasmide DNA dat codeert voor humaan interleukine 12 beoordeeld bij honden met spontane tumoren. Hoewel immuunstimulatie en anti-angiogene effecten op tumordoorbloeding meetbaar waren bij elke behandelde hond, werden geen genezingen bekomen. Bij één hond met uitzaaiingen in de iliacale lymfeknopen werd echter een

tijdelijke partiële regressie gemeten in de grootte van deze uitzaaiingen, terwijl de primaire tumor bleef groeien. Dit maakt ons hoopvol dat interleukine 12 een belangrijke rol kan spelen in het bestrijden van uitzaaiingen. Interleukine 12 is een krachtige stimulator van het aangeboren immuunsysteem en vele aangeboren immuuncellen zijn erg plastisch en oefenen pro- of antitumorale activiteiten uit naargelang hun micro-omgeving. Het is erg waarschijnlijk dat interleukine 12 de overgang van een protumorale naar een antitumorale omgeving erg positief beïnvloedt in minder uitgebouwde tumoren zoals uitzaaiingen en zeker uitzaaiingen in lymfeknopen aangezien lymfeknopen immuuncellen bevatten die kunnen reageren op interleukine 12.[430]

In Hoofdstuk 3 werden de veiligheid en antitumorale effecten van de combinatie van metronomische cyclofosfamide en een plasmide coderend voor humaan interleukine 12 (= combinatiebehandeling) beoordeeld in honden et spontane tumoren. Inflammatoire responsen werden gemeten bij elke hond na behandeling, evenals een duidelijke daling in tumordoorbloeding. Terwijl in de interleukine 12-studie de snelheid van de tumordoorbloeding slechts tijdelijk daalde, veroorzaakte de combinatiebehandeling echter een voortdurende daling in de snelheid van de tumordoorbloeding. Ondanks het gebrek aan genezingen, geven deze aanhoudende anti-angiogene effecten aan dat de algemene overleving van deze honden waarschijnlijk gestegen was na de combinatiebehandeling, hetgeen gelijkaardig was aan de bevindingen in de preklinische muizenstudie. Daarbovenop kan de combinatiebehandeling een palliatieve of ondersteunende rol spelen voor kankerpatiënten aangezien gewichtstoename of stabilisatie gemeten werd bij alle behandelde honden terwijl voor de behandeling progressief gewichtsverlies vastgesteld werd.

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CURRICULUM VITAE

Laetitia Cicchelero werd geboren op 15 mei 1985 in Laken. Ze behaalde haar diploma secundair onderwijs (Latijn-Wetenschappen) aan het Sint-Jozefsinstituut in Ternat en startte hierna in 2003 met de studies Diergeneeskunde aan de UGent. In 2009 behaalde ze het diploma van dierenarts met onderscheiding, evenals het FELASA C-statuut voor proefdierwetenschappen.

In het daaropvolgende 2,5 jaar werkte zij als voltijds dierenarts in een eerstelijnspraktijk voor gezelschapsdieren. Geboeid door het wetenschappelijk onderzoek, startte zij in juli 2012 een doctoraatstudie bij de vakgroep Voeding, Genetica en Ethologie, waar zij gedurende 3,5 jaar onderzoek verrichtte aangaande immunotherapie tegen kanker. Daarnaast was ze ook actief als lid van de Dierenwelzijnscel. De eerste 3,5 jaren werden gefinancieerd door een beurs van het Fundamenteel Wetenschappelijk Onderzoek (FWO). De laatste onderzoeksmaand werd betaald via een beurs van de Facultaire Commissie Wetenschappelijk Onderzoek (FCWO).

Laetitia Cicchelero is auteur en mede-auteur van verschillende wetenschappelijke publicaties in nationale en internationale tijdschriften. Ze nam deel aan verschillende nationale en internationale congressen en behaalde in 2014 een prijs voor beste stormsessiepresentatie op Oncopoint.

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  **Co-last and co-corresponding authors*

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- **Cicchelero L**, Vanderperren K, Denies S, Stock E, Haers H, Van Brantegem L, de Rooster H*, Sanders NN*. Contrast-enhanced ultrasound imaging of the anti-angiogenic effects of interleukin 12 in pet dogs with spontaneous malignant tumors. The 21th European Symposium on Ultrasound Contrast Imaging, Rotterdam (The Netherlands) 2016, 35-39. Oral presentation.
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